Herpesviruses and morbilliviruses of aquatic and terrestrial carnivores

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Herpesvirussen en morbillivirussen
van zeezoogdieren en op het land levende vleeseters

Proefschrift

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Those that hold
that all things are governed by fortune had not erred,
had they not persisted there.

Sir Thomas Browne
(cited after: Joseph Conrad, Chance, 1913)

Für Michaela, Marcel und Nora

List of abbreviations

AF	Antibody affinity
BHV	Bovid herpesvirus
CDV	Canine distemper virus
CHV	Canid herpesvirus
CNS	Central nervous system
Con A	Concanavalin A
CrFK	Crandell-Rees feline kidney cells
CTL	Cytotoxic T cell
DMEM	Dulbecco's modified Eagle's medium
DMV	Dolphin morbillivirus
EBV	Epstein-Barr virus
EBNA-1	Epstein-Barr virus nuclear antigen-1
EHV	Equid herpesvirus
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
F	Fusion (-gene, -protein)
FCS	Fetal calf serum
FFU	Focus-forming units
FHV	Felid herpesvirus
Gp	Glycoprotein
H	Haemagglutinin (-gene, -protein)
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HRPO	Horseradish peroxidase
HSV-1	Herpes simplex virus-1
HVS-2	Herpesvirus saimiri-2
IE	Immediate early regulatory proteins
IL-2	Interleukin-2
IPMA	Immune-peroxidase monolayer assay
kb	Kilo base pairs
KN	Kinetic neutralization
L	Large (-gene, -protein)
LMP-2	Epstein-Barr virus late membrane protein-2
M	Matrix (-gene, -protein)
MAb	Monoclonal antibody
MDCK	Madin Darby canine kidney cells
ML	Modified-live attenuated (vaccine)
MOI	Multiplicity of infection
MV	Measles virus

N	Nucleocapsid (-gene, -protein)
nAb	Neutralizing antibody
ND_{50}	Neutralizing dose 50% endpoint
NK	Natural killer cells
ODE	Old dog encephalitis
ONST	CDV vaccine strain 'Onderstepoort'
ORF	Open reading frame
P	Phospho (-gene, -protein)
PBMC	Peripheral blood mononuclear cell
PBST	Phosphate-buffered saline supplemented with Tween 20
PDV	Phocid distemper virus
PHA	Phythaemagglutinin
PhHV	Phocid herpesvirus
PLA	Peroxidase-linked antibody assay
PMV	Porpoise morbillivirus
PPRV	Peste-des-petits-ruminants virus
ROC	CDV vaccine strain 'Rockborn'
RPV	Rinderpest virus
SeHV-1	Seal herpesvirus-1
SeKC	Seal kidney cells
SHV-1	Suid herpesvirus-1 (pseudorabies virus)
SPF	Specific pathogen-free
SSPE	Subacute sclerosing encephalitis
$TCID_{50}$	Tissue culture-infectious dose 50% endpoint
U_l	Unique long fragment
U_s	Unique short fragment
UTR	Untranslated region
VF	Visual fields
VZV	Varicella zoster virus
wt	Wild-type

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Chapter 1

General introduction

Section 1.1

Aspects of herpesvirus biology

Taken in part from:

Harder, T.C., Plötz, J., Liess, B. (1991a).
Antibodies against European phocine herpesvirus isolates detected in sera of Antarctic seals.
Polar Biol. 11, 509–512.

Introduction

The *Herpesviridae* represent a family of diverse and complex viruses of vertebrates (Roizman *et al.*, 1992; Roizman, 1996) and are believed to be of comparatively ancient origin (Karlin *et al.*, 1994a; McGeoch *et al.*, 1995). Many mammalian species are host to more than one herpesvirus species. In humans, e.g., an eighth distinct herpesvirus species has recently been discovered (Chang *et al.*, 1994; Moore *et al.*, 1996). Disease associated with *lytic* herpesvirus infection in their natural hosts varies considerably from mild, superficial mucocutaneous lesions, acute respiratory disease, benign lymphoproliferative disorders to fatal generalized infections and congenital malformations (Peterslund, 1991). Besides certain viral factors, immuno-(in)competence of the natural host constitutes a major pathogenic factor (Fawl & Roizman, 1994). Some herpesviruses also display transforming properties (e.g. Meinl *et al.*, 1995; Thorley-Dawson *et al.*, 1996) or act as co-factors in tumorigenesis (Nazarin, 1979; Khanna *et al.*, 1995; Mesri *et al.*, 1996). Herpesviruses characteristically establish life-long *latent* infections in their hosts (Stevens, 1994). Periodically, latent (silent) virus can become reactivated leading to limited lytic replication and shedding of infectious particles. Latently infected hosts, therefore, represent the major herpesvirus reservoir.

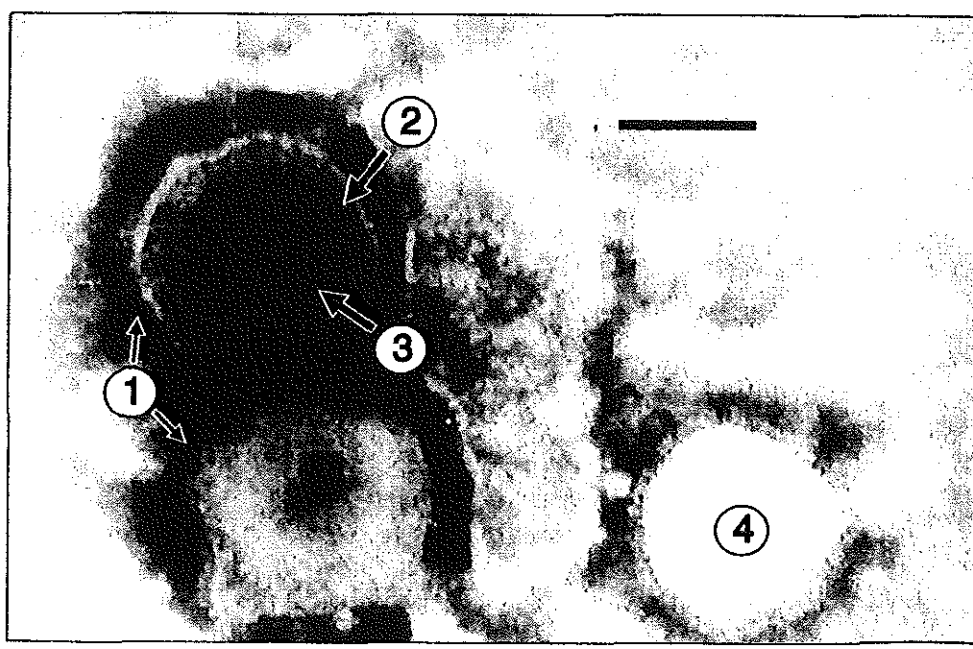

Figure 1. Electronmicroscopic photography of phocid herpesvirus type-1 (courtesy of Dr. J.S. Teppema, RIVM Bilthoven, The Netherlands). Bar indicates 100 nm. 1 – Lipid envelope with spikes; 2 – Tegument; 3 – Capsid; 4 – Intact virion, outer surface with glycoprotein projections.

Table 1. *Herpesviridae* — Taxonomy and genome properties of some herpesviruses of medical and veterinary concern (modified from Roizman *et al.*, 1992).

Official designation	Common name	Genome properties		
		G+C%	Type	Size (kB)
α-Herpesvirinae[1]				
Simplexvirus[2]				
Human herpesvirus-1	Herpes simplex virus-1	67	E	152
Human herpesvirus-2	Herpes simplex virus-2	69	E	152
Varicellovirus				
Human herpesvirus-3	Varicella-zoster virus	46	D	125
Equine herpesvirus-1	Equine abortion virus	57	D	150
Suid herpesvirus-1	Pseudorabies virus	74	D	140
Felid herpesvirus	Feline rhinotracheitis virus	46	D	134
Canid herpesvirus	Canine herpesvirus	32	–	–
Not assigned to genus.				
Gallid herpesvirus-2	Marek's disease virus	47	E	180
β-Herpesvirinae				
Cytomegalovirus				
Human herpesvirus-5	Human cytomegalovirus	57	E	229
Muromegalovirus				
Murid herpesvirus-1	Murine cytomegalovirus	59	F	235
Roseolovirus				
Human herpesvirus-6	–	42	A	162
Human herpesvirus-7	–	44	A	140 - 160
γ-Herpesvirinae				
Lymphocryptovirus (γ_1)				
Human herpesvirus-4	Epstein-Barr virus	60	C	172
Rhadinovirus (γ_2)				
Saimirine herpesvirus-2	Herpesvirus saimiri	46	B	155
Bovid herpesvirus-4	–	50	B	145
Alcelaphine herpesvirus-1	Malignant catarrhal fever virus	61	B	160
γ_3 – *herpesvirus*[3]				
Equine herpesvirus-2	–	57	A	192

[1] Subfamily,

[2] Genus,

[3] proposed by Telford *et al.*, 1993,

– no data available.

Herpesvirus classification

Membership in the *Herpesviridae* family is substantiated primarily on basis of virion morphology (Rixon, 1993). Herpesviruses form quasi-sphaerical particles ranging in diameter between 150 - 200 nm (Fig. 1). The outer host cell-derived lipid bilayer is spiked with several viral glycoprotein (gp) and non-gp transmembrane proteins. Underneath the envelope the tegument, an amorph proteinaceous mass, is located. An icosahedric capsid of 100 - 110 nm in diameter is embedded within the tegument and harbours a double-stranded DNA genome. At least 33 proteins are known to contribute to the virion architecture of the human herpes simplex virus-1 (HSV-1; Roizman & Sears, 1996).

Three subfamilies of herpesviruses (the α-, β- and γ-*Herpesvirinae*) are distinguished mainly according to certain biological characteristics (duration of the replication cycle, host cell tropisms and location of latency). Nucleotide sequence data, available for a growing number of herpesviruses species, confirmed and justified this classification in most cases. Table 1 presents a brief overview of the taxonomic status and the molecular properties of some herpesviruses of medical and veterinary concern.

Genome organization

The size of the double-stranded DNA genome of different herpesviruses species varies widely between 125 to 235 kB, and so does the G+C content which ranges between 32% to 74% (Tab. 1). Six different genome types (A–F) are distinguished on basis of presence and arrangement of reiterated sequences in relation to unique sequences. The genomes comprise a coding capacity ranging from approximately 70 (α-herpesviruses) to up to 200 (β-herpesviruses) genes (McGeoch, 1989). Genes are densely packed in a consecutive order along the genome and are either separated by short intergenic sequences or overlap slightly (Davison, 1993). Open reading frames (ORFs) are located on both strands. A core of about 40 genes arranged in several characteristic blocks is conserved between all herpesviruses. Families of related genes described, e.g., for Epstein-Barr virus, EBV (Baer *et al.*, 1984), human cytomegalovirus, HCMV (Weston & Barrell, 1986) or in the unique short (Us) region of α–herpesviruses (McGeoch, 1990) are believed to have arisen by gene duplication and divergence from ancient progenitors of presumably non-herpesviral origin.

Lytic replication

Viral integral membrane glycoproteins (gps) mediate both attachment to receptor molecules at the host cell surface and penetration via fusion of viral and cellular membranes at neutral pH values (reviewed by Spear, 1993). At least ten gps have been identified in HSV-1 infected cells, many of which have also been detected in the virion envelope. Along with their location as integral proteins in the virion envelope and - at least for some - also in the infected host cell membrane, the gps are major targets of the immune surveillance. Certain gp 'families' (e.g. gB, gH and gL) are encoded by members of all herpesvirus subfamilies

Table 2. Herpes simplex virus-1 glycoproteins and their equivalents in canid (CHV) and felid (FHV) herpesviruses (modified from Crabb & Studdert, 1995).

HSV glycoprotein				Homologues	
Designation	Gene[1]	Ascribed function	Essential[2]	CHV[3]	FHV[4]
gB	UL27	Penetration, fusion	+	gB	gB
gC	UL44	Adsorption, C3b receptor	—	gC	gC
gD	US6	Penetration, fusion	(+)	gD	gD
gE	US8	Fc receptor (complex with gI)	—	n.d.	gE
gG	US4	n.d.	—	n.d.	gI
gH	UL22	Penetration, fusion, complex with gL	+	n.d.	gH
gI	US7	Fc receptor (complex with gE)	—	n.d.	gI
gJ[5]	US5	n.d.	—	n.d.	n.d.
gK	UL53	Fusion	—	n.d.	n.d.
gL	UL1	see gH	+	n.d.	n.d.
gM	UL10	n.d.	—	n.d.	n.d.

[1] Numbering according to McGeoch *et al.*, 1988,

[2] Essentially required for replication in cell cultures,

[3] Limbach *et al.*, 1994; Remond *et al.*, 1996,

[4] Maeda *et al.*, 1990; Maeda *et al.*, 1991; Spatz *et al.*, 1994; Willemse *et al.*, 1995,

[5] Predicted from the HSV-1 nucleotide sequence. n.d. — no data.

analysed so far and were found to be indispensable for viral replication in cell cultures; other gp-encoding genes can be deleted while the mutant remains replication-competent (Spear, 1993; Mettenleiter *et al.*, 1994). However, wild-type (wt) isolates in general retain all of the 'non-essential' gps indicating that these gps confer a certain selective advantage *in vivo*. Table 2 comprises a list of HSV-1-encoded gps and their attributed functions. Gps identified up to now in herpesviruses of terrestrial carnivores are indicated for comparison.

Insight into the molecular events associated with attachment of herpesviruses to their target cells and knowledge of the interacting receptors and ligands which are diverse for the different herpesvirus subfamilies is still limited (Fingeroth *et al.*, 1984; WuDunn & Spear, 1989; Karger & Mettenleiter, 1993; Norkin, 1995; Zhu *et al.*, 1995; Montgomery *et al.*, 1996; Whitbeck *et al.*, 1997). Following penetration by fusion at neutral pH values at the cell membrane, the capsid/tegument complex is transported through the cytoplasm and viral DNA along with at least a part of the tegument proteins is released through the nuclear pores. Circularization or concatemerization of the genome(s) precedes initiation of the transcription process. Herpesviruses encode a strong α-*trans*-inducing factor (a tegument protein) which, in co-operation with cellular transcription factors, triggers transcription of immediate early (IE or α-) genes. Their products, in turn, further control a tightly ordered temporal cascade of gene expression involving a series of *cis*- and *trans* -acting elements (Batterson & Roizman, 1984; Hay & Ruyechan, 1992). During the early or β-phase, the viral nucleic acid metabolism and DNA replication machinery is activated. Virion DNA is replicated in the nucleus by a

rolling circle mechanism yielding concatemeric progeny genomes in a head-to-tail order. Proteins synthesized in the late or γ-phase fulfil mainly architectural functions. Capsids, while assembling in the nucleus, are loaded with a single-unit genome copy. Encapsidated DNA appears to exist in a liquid crystalline state (Booy *et al.*, 1991). Packaged capsids are released from the nucleus into the cytoplasm. The exact pathways involved in tegumentation, envelopment and final egress of virions are still a matter of debate (Roffman *et al.*, 1990; Torrisi *et al.*, 1992; Rixon, 1993). A lytic replication cycle of α-herpesviruses is comparatively short and always associated with rapidly spreading, extensive cytolytic destruction in permissive cell cultures. Replication of β- and γ-herpesviruses, in contrast, is much more delayed and often cytopathic alterations are less striking.

Lytic replication of herpesviruses inevitably results in death of the host cell. However, many target cells of herpesviruses maintain vital functions and are postmitogenically fixed (e.g. neurons). Loss of such cells cannot be compensated. The host-herpesvirus relationship, therefore, must be meticulously balanced to an equilibrium. Consequently, exacerbation from these intricate mechanisms may result in generalized, fatal disease which can be seen in susceptible aberrant host species and in immuno-immature (fetus, neonate) or immunocompromised individuals of the natural host species.

Latency

Establishment, maintenance and periodical reactivation from presumably life-long persisting latent infection are hallmarks of herpesvirus biology (Rock, 1993). Initiation of latent infection is assumed to be an early event after penetration and should occur before efficient transcription of IE genes is launched. Sites and cell types where herpesviruses establish latent infection as well as the molecular events associated with latency vary between members of the different subfamilies: α-herpesviruses establish latent infections predominantly in neuronal cells. Lymphoid cell types are targets of γ-herpesvirus latent infections. Little is known about latency of β-herpesviruses. At present it can not even be excluded that so-called 'latent' β-herpesvirus infections instead are chronic productive infections at a very low level (Koszinowski *et al.*, 1994). Whatever the mechanisms, during latency the virus efficiently escapes from immune surveillance.

Cells that are latently infected by herpesviruses appear to contain several copies of the viral genome in an episomal form (Stevens, 1994). Gene expression of α−herpesviruses apparently falls completely silent during latency, since the so-called latency-associated transcripts (LATs) probably remain untranslated (McGeoch *et al.*, 1993; Feldman, 1994). In contrast, at least two viral proteins (EBNA-1 and LMP2) are expressed in resting B cells which constitute a reservoir of latent EBV infection (Thorley-Lawson *et al.*, 1996). In spite of the fact that these proteins are potent immunogens and targets of cytotoxic T cells (CTLs), the cells survive.

The molecular events associated with the reactivation process are not fully understood. Experimentally, latent herpesvirus infections have been succesfully reactivated by an array of measures including local traumas, social stress or glucocorticoid application. Clinical symptoms during a reactivation phase (recurrent infections) are generally mild or even asympto-

matic while still significant titers of infectious virus can be shed (Enquist, 1994). As such, latently infected hosts, so-called 'carriers', create the most crucial epidemiological impact.

Host immune responses

Upon primary herpesvirus infection, humoral and cellular immune responses are induced which - at least in immuno-competent natural hosts - confine lytic virus replication locally and ensure virus elimination from these sites. However, neither establishment of latency nor re-infections are prevented. Instead, herpesviruses have evolved a plethora of sophisticated immune evasion mechanisms which have only partially been unraveled (Bielefeldt-Ohmann & Babiuk, 1988; Hsu *et al.*, 1990; Gregory *et al.*, 1991; Banks & Rouse, 1992; Koszinowksi *et al.*, 1994; Hill *et al.*, 1995; Levitskaya et al., 1995; Burgert, 1996; Hengel *et al.*, 1996).

Host immune responses are stimulated and directed against an array of viral proteins of which IE regulatory proteins and viral membrane gps appear to hold a prominent place. In particular, gps of the gB, gC and gD family have been shown to induce neutralizing antibodies as well as cytotoxic T cells (Para *et al.*, 1985; Witmer *et al.*, 1990; Eis-Hubinger *et al.*, 1993; Ghiasi *et al.*, 1994; Pereira, 1994; Tewari *et al.*, 1994). While CTLs seem to be pivotal in the elimination of virus-infected cells following primary infection, antibodies are believed to play an important role in limiting spread of virus after reactivation from latency (Nash & Wildly, 1983; Simmons & Nash, 1989; Rinaldo *et al.*, 1993). Reactivation from latency is not inhibited by a sustained herpesvirus-specific immune response.

Vaccine development

Major obstacles hampering the development of efficacious and safe herpesvirus vaccines are the complexity of these viruses and, in particular, their ability to establish life-long latent infections. The ideal herpesvirus vaccine – which as yet is not available – would not only prevent disease following primary infection by wt virus, but would also block the establishment of latency. However, if clinical disease following primary infection poses the major problem of a certain herpesvirus infection, then sole protection against disease still is a desirable option (Inglis, 1995).

Modified-live attenuated (ML) herpesviral vaccines for use in humans have not been licensed yet (Burke, 1993) with the single exception of a Varicella zoster virus (VZV) vaccine (Oka strain), originally designed to protect leukemic children from lethal chicken pox (Bergen *et al.*, 1990; Gershon, 1995). In veterinary medicine, in contrast, ML herpesviral vaccines are widely used in the prevention of α-herpesvirus induced diseases of both livestock and pet animals. Marker vaccines featuring genetically engineered deletion mutant strains which are phenotypically attenuated are gradually replacing traditional vaccine strains and allow serologic discrimination of vaccinated and wt virus infected animals (Van Oirschot *et al.*, 1990; Van Drunen Littel-van den Hurk *et al.*, 1993; Mettenleiter *et al.*, 1994). These vaccines have proven quite efficient in prevention of disease, but they do not inhibit wt virus infection under field conditions and establish latent infections themselves. Nevertheless, vaccinated animals

were shown to have less frequent episodes of reactivation and shed lower titers of virus for a shorter period as compared to non-vaccinated controls (reviewed by Young & Smith, 1995).

To circumvent the above mentioned problems of ML herpesvirus vaccines, recombinant subunit vaccines have been evaluated and succesfully tested in several animal models and for use in humans (Morein & Merza, 1991; Langenberg *et al.*, 1995).

Herpesvirus infections of aquatic and terrestrial carnivores

While herpesvirus infections and their association with respiratory, ocular and reproductive diseases in dogs and cats have been well known for a long time, the earliest report of herpesvirus infections in aquatic carnivores only originates from 1984 when an α-herpesvirus was found the cause of a major disease outbreak among newborn harbour seals (*Phoca vitulina*) in a Dutch seal sanctuary (Osterhaus *et al.*, 1985; Borst *et al.*, 1986): Mortality rates peaked at about 50%. Fatal cases were mainly attributed to generalized infections causing focal hepatitis, nephritis and encephalitis. In milder cases, the symptoms were restricted to the upper respiratory tract, occasionally leading to pneumonia. Herpesvirus isolates recovered from these cases and originally designated seal herpesvirus-1 (SeHV-1) were shown to be serologically closely related to the authentic α-herpesviruses of dogs (canid herpesvirus, CHV) and cats (felid herpesvirus, FHV) (Osterhaus *et al.*, 1985). SeHV-1, now referred to as phocid herpesvirus (PhHV) was also shown to replicate in cells of feline origin emphasizing the close relationship of these viruses and giving rise to speculations concerning mutual interspecies transmission (Harder *et al.*, 1991a; Stenvers *et al.*, 1992a). Serosurveys showed that PhHV or closely related herpesvirus species are prevalent in several pinniped species worldwide including also those of Arctic and Antarctic habitates (Vedder *et al.*, 1987; Harder *et al.*, 1991a; Stenvers *et al.*, 1992b; Stuen *et al.*, 1995).

In the course of the 1988 seal mass mortality, an epizootic caused by the formerly undescribed phocid distemper morbillivirus (PDV) which resulted in loss of approximately 17.000 seals in northwestern European waters, PhHV was initially suspected a primary causative agent (Osterhaus, 1988). Later on, the generalized PhHV infections frequently seen in moribund seals of all ages clearly had to be interpreted as a sequel of a severe immunosuppression owing to an underlying PDV infection (Osterhaus *et al.*, 1988). However, co-infections with PhHV were considered to have signficantly contributed to the high mortality rates, particularly among adult harbour seals (Zhang *et al.*, 1988; Stenvers *et al.*, 1992a). Likewise, aggravation of acute canine distemper and measles cases by canine herpesvirus and herpes simplex virus infections, respectively, have been documented (Orren *et al.*, 1981; Harder, unpublished).

Section 1.2

Aspects of morbillivirus biology

Taken in part from:

Harder, T.C., Willhaus, T., Frey, H.-R., Liess, B. (1990).
Morbillivirus infections of seals during the epizootic in the Bay of Heligoland 1988.
III. Transmission studies of cell culture-propagated phocine distemper virus
in harbour seals *(Phoca vitulina)* and a grey seal (*Halichoerus grypus*).
J. Vet. Med. B 37, 641–650.

Harder, T.C., Moennig, V., Greiser-Wilke, I., Barrett, T., Liess, B. (1991).
Analysis of antigenic differences between sixteen phocine
distemper virus isolates and other morbilliviruses.
Arch. Virol. 118, 261–268.

Harder, T.C., Willhaus, T., Leibold, W., Liess, B. (1992).
Investigations on course and outcome of phocine distemper virus infection in harbour seals
(*Phoca vitulina*) exposed to polychlorinated biphenyls: Virological and serological results.
J. Vet. Med. B 39, 19–31.

Harder, T.C., Stede, M., Willhaus, T., Schwarz, J., Heidemann, G., Liess, B. (1993).
Morbillivirus antibodies of maternal origin in harbour seal pups (*Phoca vitulina*).
Vet. Rec. 132, 632–633.

Introduction

Taxonomically considered, morbilliviruses are a genus of the *Paramyxoviridae* family (Pringle, 1994): The agent causing measles in humans is the type species of this genus which up to now comprises five further members all of which infect animal hosts (Tab. 1). Morbilliviruses consist of a non-segmented linear single-stranded RNA genome of negative polarity comprising about 15900 bp. The RNA is enclosed in a helical nucleocapsid formed by the N protein. In addition, mature ribonucleoprotein complexes also contain copies of the phospho- (P) and large (L) proteins. The host cell-derived lipid envelope is spiked with transmembrane haemagglutinin (H) and fusion (F) glycoproteins. Internally, the envelope is stabilized by a layer of the matrix (M) protein. The diameter of virions varies between 150 to 250 nm (Fig. 1).

Morbilliviruses are highly contagious and transmission occurs predominantly via aerosols (Appel, 1969; Black, 1991). In susceptible hosts, an acute febrile and multisystemic disease is induced (Appel *et al.*, 1981). Induction of severe immunosuppression is a hallmark of morbillivirus infections. In addition, the morbilliviruses of carnivores are also highly neurotropic and capable of inducing chronic persistent infections of the central nervous system (CNS) (Vandevelde & Zurbriggen, 1995). Once introduced into a naive host population, morbilliviruses provoke a rapidly spreading epidemic associated with high mortality rates in hosts of all age classes. Provided a surviving host population of sufficient size and density, morbilliviruses will establish endemic infections where disease is usually restricted to offsprings with declining maternal immunity (Black, 1966). The human population clearly fulfils the criteria to allow perpetuation of measles virus (MV). Measles, therefore, will continue to account for more than a million deaths of children annually in underdeveloped countries unless an extensive vaccination coverage is guaranteed (Black, 1991; Rota *et al.*, 1996).

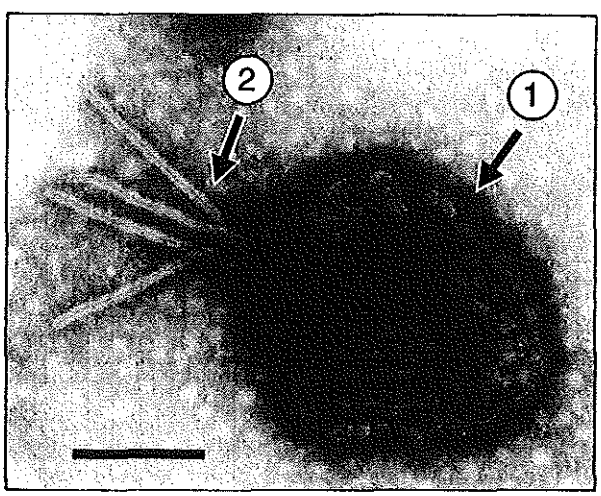

Figure 1. Electronmicroscopic photography of phocid distemper virus (PDV) (courtesy of Dr. J.S. Teppema, RIVM Bilthoven, The Netherlands). Bar indicates 100 nm. 1 – Lipid envelope, spiked with H and F glycoproteins; 2 – Nucleocapsid, protruding from disrupted virion.

Table 1. *Morbilliviruses* — Taxonomy, host spectrum and clinical impact.

Morbillivirus species	Natural host spectrum	Associated diseases
Measles virus (MV)	Humans (Primates)	Measles; giant cell pneumonia; subacute sclerosing panencephalitis; inclusion body encephalitis
Rinderpest virus (RPV)	*Artiodactyla*	Cattle plague
Peste-des-petits ruminants virus (PPRV)	*Artiodactyla*	Pneumoenteritis of sheep and goats
Cetacean distemper virus[1]	*Cetacea*	Distemper
Canine distemper virus (CDV)	*Carnivora*[2]	Distemper; osteodystrophia; old dog encephalitis (ODE)
Phocid distemper virus (PDV)	*Pinnipeda*	Distemper

[1] Includes dolphin distemper virus (DMV), and porpoise morbillivirus (PMV),

[2] Notable exceptions until 1994: *Felidae, Ursidae.*

Genome organization

Close to its 3' end, the morbillivirus genome harbours a promotor region where binding of the virion-derived RNA-dependent RNA polymerase, composed of the L and, probably also of the P protein, is initiated (Blumberg *et al.*, 1988). Transcription of mRNAs proceeds in a predominantly monocistronic pattern from gene to gene which are in the order 3'-UTR-N-P(C,V)-M-UTR-F-H-L-UTR-5' (UTR - untranslated regions). A nontranscribed consensus intergenic trinucleotide separates sequence motifs utilized for polyadenylation and for re-initiation of transcription, respectively, of adjacent genes (Crowley *et al.*, 1988). Between the ORFs of the M and F genes an unusually long UTR (up to 1.0 kb) is located whose function is not well understood (Hasel *et al.*, 1987; Evans *et al.*, 1990; Cathomen *et al.*, 1995). Owing to a certain likelihood of dissociation of the transcriptase complex at each gene junction, a gradient of mRNA abundancies from the most 3' (N) gene to the most 5' (L) gene is generated whereby synthesis of the different viral proteins is economized according to the amounts required (Barrett *et al.*, 1991). Apart from rare read-through transcripts, all mRNAs, except for the P gene transcript, are monocistronic. The P mRNA codes for at least three proteins: The P protein itself is encoded by the longest ORF of the P gene (Barrett *et al.*, 1985). The small non-structural C protein is generated from an alternate reading frame (Bellini *et al.*, 1985). A second non-structural protein, termed V, is derived from an edited P mRNA in which an untemplated G residue is inserted at a defined position shifting the reading frame of the distal quarter of the P mRNA (Cattaneo *et al.*, 1989; Haas *et al.*, 1995). V and P proteins, therefore, share identical N-terminal amino acid sequences (Martens *et al.*, 1995).

Positive-stranded antigenomic full-length RNA which, after encapsidation, serves as a template for genome replication is produced late in the infectious cycle and is dependent on a critical amount of progeny N and P protein in the infected cell.

Virus replication

Selection of and attachment to host cells is mediated by the H protein (Stern *et al.*, 1995). The membrane co-factor CD46 – possibly in complex with moesin molecules – acts as a cellular receptor for MV on human cells (Naniche *et al.*, 1993; Dunster *et al.*, 1994; Schneider-Schaulies *et al.*, 1995a). Interestingly, MV strains exhibiting an attenuated phenotype, in contrast to virulent isolates, efficiently down-regulate membrane CD46 (Schneider-Schaulies *et al.*, 1995c; Bartz *et al.*, 1996). Cells which become devoid of CD46, however, are rendered more susceptible to complement-mediated lysis. *In vivo*, these cells can be expected to be more rapidly cleared: an effect that might contribute to the pathogenicity of MV (Schnorr *et al.*, 1995). A reverse genetic system, recently developed for MV, will further aid in pinpointing virulence determinants (Radecke *et al.*, 1995). Recently, the CD9 equivalent has been identified as a co-factor of receptor-mediated entry of CDV whereby an as yet unknown 65 - 75 kD protein has been suspected to represent the primary cellular receptor (Löffler *et al.*, 1997).

Upon receptor interactions, sterical processes expose the fusion domaine, a highly hydrophobic region of the F protein, which mediates fusion with the cellular membrane at neutral pH values (Wild & Buckland, 1995). All subsequent steps of replication occur in the cytoplasm. Several host cell proteins have been identified as possible co-factors for morbillivirus replication (Moyer *et al.*, 1990; Oglesbee *et al.*, 1996). The role of the non-structural C and V proteins remains to be fully elucidated (Wardrop & Briedis, 1991; Radecke & Billeter, 1996). Posttranslational modifications of morbillivirus proteins include phosphorylation (N, P, V, possibly L) and N-linked glycosilation in the exocytic pathways (H and F). Further maturation of the F protein essentially consists of endoproteolytic cleavage at a furin-sensitive consensus sequence yielding two disulfide-linked fragments (F_1–F_2). The new N-terminus of the F_1 fragment represents the fusion domaine (Morrison & Portner, 1991). Virions which have exclusively incorporated uncleaved F proteins are non-infectious . Virus progeny is released by budding from the cell surface.

Pathogenesis and immune response

Primary target cells following aerosol transmission of morbilliviruses are found in the respiratory epithelia and may include descendants of the monocyte/macrophage lineage (Appel, 1969; Esolen *et al.*, 1993). Centripetal dissemination of progeny virus via tributary lymphnodes establishes infection of central lymphatic tissues. Virus is redisseminated from these tissues via a cell-associated viremia toward epithelia of various organs. Virus is excreted with all body secretions. Severe immunopathological changes which have been attributed, at least in part, to lytic morbillivirus replication in lymphoid and myeloid cells determine a global immunosuppression (Krakowka *et al.*, 1980; Casali *et al.*, 1989). More recent data obtained for MV suggest a role of decreased IL-12 production by monocytes which is downregulated by signal transduction via the CD46 molecule following binding of the MV H-protein (Karp *et al.*, 1996). Development of opportunistic infections which even may occlude the underlying primary cause, contribute significantly to mortality rates in the course of morbillivirus

epizootics (Beckford *et al.*, 1985).

Acute morbillivirus-induced disease is manifested at the respiratory (MV, CDV, PDV, DMV, PPRV) and at the gastrointestinal tract (CDV, RPV, PPRV) (Yamanouchi, 1980). Skin affections have been described in MV and CDV infections, including a maculo-papular rash characteristic of MV infection in humans (Griffin & Bellini, 1996). Except for RPV and PPRV infections, involvement of the CNS is another hallmark of morbillivirus infections. CDV and PDV are known to exhibit a possibly strain-dependent neuroinvasiveness leading to acute encephalomyelitis in an estimated 20 - 70% of naturally occuring distemper cases (Blixenkrone-Møller *et al.*, 1993). MV-associated encephalomyelitis occurs at strikingly lower frequencies (0.1%) (Johnson *et al.*, 1984; Schneider-Schaulies *et al.*, 1995b). In addition, CDV is also known to be able to establish fatal chronic persistent infection of the CNS which is characterized by widespread and massive demyelinisation of both white and gray matters (Vandevelde & Zurbriggen, 1995). More subtle changes, due to persistent CDV infections of the CNS and leading to progressive motor and behavorial alterations, have been described as old dog encephalitis (ODE) which may be similar to a rarely described chronic persisting MV CNS infection in humans, correlated with subacute sclerosing panencephalitis (SSPE) (Lincoln *et al.*, 1971).

Survivors of natural morbillivirus infections mount a life-long protective immunity which is based on neutralizing antibodies (nAbs), directed against the H and F gps, and on CTLs, specific for various viral protein targets (Norrby, 1991). NAb appear crucial for the prevention of (re-)infection and for limiting virus dissemination (Chen *et al.*, 1990). The H protein is the main target of nAbs which are highly species-specific (Giraudon & Wild, 1985; Sheshberadaran & Norrby, 1986). Some neutraliziation-relevant epitopes have also been located on the F protein (Malvoisin & Wild, 1990). The majority of F-specific Abs, however, broadly cross-reacts between the different morbillivirus species and may inhibit direct cell-to-cell spread of virus by fusion (Sheshberadaran *et al.*, 1986; Sato *et al.*, 1989).

A complicated and often fatal course of MV infection in patients with pronounced T cell deficiencies in comparison to those with B cell disorders points toward a paramount importance of T cell-mediated immunity in recovery from infection (Norrby, 1991; Beauverger *et al.*, 1996; Griffin & Bellini, 1996). MV-specific CTLs of both MHC-class I and -II restriction have been detected and characterized at the clonal level (Van Binnendijk et al., 1989 & 1990).

Epidemiology and control

Morbilliviruses are transmitted horizontally although intrauterine CDV infections have been described (Krakowka *et al.*, 1977; Black, 1991). Course and outcome of morbillivirus infections are modulated by strain-dependent viral properties and by the host's immunocompetence, constitution and possibly also by its genetic background. In addition, size, density, and immune status of the respective host population are pivotal factors (Black, 1966; Aaby *et al.*, 1988). Owing to a comparatively short infectious period and their fragility outside in the environment, morbilliviruses require rapid transmission before being either eliminated by their hosts or before having eliminated the hosts themselves. This implies a dense population

to sustain an endemic morbillivirus status. The observed critical community size below which MV infections will not persist amounts to 250.000 – 400.000 (Keeling & Grenfell, 1997). Since survivors of infection in principle seem to be refractory to reinfection, morbilliviruses depend on the rate of offspring production to establish an endemic status. Among the morbilliviruses, CDV occupies the broadest spectrum of natural hosts and interspecies transmissions frequently do occur. In contrast, humans appear to constitute the only natural reservoir of MV. Eradication of MV, therefore, may be a promising perspective, provided a continuing and extensive supply of vaccines worldwide (Shepard, 1994; Rota *et al.*, 1996).

In general, modified-live (ML) morbillivirus vaccines have proven safe and efficient in preventing disease. The safety of high-dose MV ML vaccines, however, has recently been challenged by reports indicating immunosuppressive side-effects in children when administered at less than one year of age (Hussey *et al.*, 1996). With respect to a resurgence of measles and distemper, respectively, associated with a genetic and, possibly, also antigenic drift observed in currently circulating MV wt isolates, a continuing assessment of the efficacy of commercial MV and CDV vaccines concerning their protective capacities against contemporary wt strains is mandatory (Atkinson & Orenstein, 1992; Weiss, 1992; Rima *et al.*, 1995a & 1997; Rota *et al.*, 1995). Recent attempts to further improve morbillivirus vaccines include the production of recombinant animal poxviruses, ISCOM-based subunit vaccines consisting of the viral H and F membrane gps as well as synthetic peptides (Varsanyi *et al.*, 1987; Visser *et al.*, 1992; Romero *et al.*, 1995; Obeid *et al.*, 1995). None of these developments, however, has yet succeeded in replacing the traditional ML morbillivirus vaccines.

Morbillivirus infections of aquatic and terrestrial carnivores

In April 1988, unusually high mortality rates were observed among harbour seals ranging in the Danish Kattegat. The seals predominantly exhibited respiratory but also gastrointestinal and nervous symptoms. The disease spread rapidly around Skagen into the German and Dutch Wadden Sea, crossed the North Sea towards the British Islands and further into Irish waters. When in the fall of 1988 the epizootic had ceased, an estimated 17.000 seals, equivalent to 60% of the population in some areas, had succumbed to the disease (Heide-Jørgensen *et al.*, 1992). Due to the epizootic disease pattern, an infectious agent was suspected at its basis. Apart from a plethora of parasites and bacteria, several virus species were isolated from diseased seals, including picorna-, herpes- and morbilliviruses (Cosby *et al.*, 1988; Osterhaus, 1988; Osterhaus *et al.*, 1988; Liess *et al.*, 1989; Have *et al.*, 1991; Frey *et al.*, 1989). By fulfilment of the postulates of Henle and Koch, morbilliviruses were identified as the primary causative agent (Osterhaus *et al.*, 1988, 1989a; Harder *et al.*, 1990, 1992; Visser *et al.*, 1992). Concerted follow-up antigenic and molecular studies allowed the classification of the so-called seal morbillivirus as a separate, previously unknown morbillivirus species, now designated *phocid* distemper virus, PDV (Kennedy *et al.*, 1988a; Örvell *et al.*, 1990; Visser *et al.*, 1990; Haas *et al.*, 1991; Kövamees *et al.*, 1991a). CDV represents the closest relative of PDV among the morbilliviruses (Blixenkrone-Møller, 1993). Later on, PDV was shown to be endemic in many seal populations worldwide including Arctic and Antarctic species (Bengtson *et al.*,

1991; Henderson *et al.*, 1992; Markussen & Have, 1992; Ross *et al.*, 1992). Introduction of PDV by migrating Arctic seals into the European harbour seal population, until then naive to morbilliviruses, would be a plausible but, to this end, unprovable explanation for the 1988 epizootic (Dietz *et al.*, 1989). Environmental pollution was of major public concern as a possible aggravating co-factor of the epizootic. Careful longitudinal studies of two seal cohorts being fed on fish from the Northern Atlantic or from the comparatively highly contaminated Baltic Sea indeed demonstrated negative impacts of organochlorine burdens on the immune system of seals, with impaired T cell responses and lowered NK cell activities being most notable (De Swart *et al.*, 1995; Ross *et al.*, 1996).

Consecutive virological studies led to the discovery of further new members of the morbillivirus genus (dolphin and porpoise morbillivirus), responsible for epizootics associated with high mortality rates among cetacean species in the Mediterraenean Sea in 1990/1991 (striped dolphin, *Stenella coeruloalba*) and in the North Sea (harbour porpoise, *Phocoena phocoena*) in 1990, respectively (Kennedy *et al.*, 1988b; Domingo *et al.*, 1990; Barrett *et al.*, 1993; Visser *et al.*, 1993a).

Chronologically in parallel with the European seal mass mortality, a similar epizootic was reported from Siberian Lake Baikal seals (*Phoca sibirica*) which were also found to die from a morbillivirus infection (Grachev *et al.*, 1989; Osterhaus *et al.*, 1989b). The agent in these cases, however, proved to be a true strain of CDV (Visser *et al.*, 1993b; Mamaev *et al.*, 1995). Any epidemiological links to the European seal epizootic, therefore, were ruled out. CDV infections of aquatic mammals probably were not unprecidented according to a report in which a distemper-like disease was described in crabeater seals (*Lobodon carcinophagus*) off the Antarctic coast in 1955; simultaneously, canine distemper occured in sled dogs brought to the same area of Antarctica (Laws & Taylor, 1957).

A recent and most unexpected morbillivirus epizootic among wildlife carnivores was reported in 1994 from lions (*Panthera leo*), ranging in the Tanzanian Serengeti National Park: up to 30% of the population were killed (Morell, 1994). Before, small scale outbreaks of distemper-like disease have occured in big cats kept in North American zoos (Appel *et al.*, 1995). While the identity of the Serengeti lion morbillivirus remained obscure, the outbreaks in the US exotic *Felidae* have serologically been linked to CDV-like viruses.

Aim and outline of the thesis

With respect to their biology, herpesviruses and morbilliviruses hold almost diametrically opposed positions within the spectrum of viruses. However, during the last decade, these viruses created the most significant impact on aquatic and – in addition to the canine parvovirus pandemia and the feline immunodeficiency virus infections – also on wildlife terrestrial carnivores. This study aims at the characterization of these viruses at the molecular level in order to elucidate the phylogenetic and epidemiological relationships of herpes- and morbilliviruses in land-based and aquatic carnivores, respectively.

Despite the evidence that potentially highly pathogenic herpesviruses (SeHV) are prevalent worldwide in many pinniped populations, some of them highly endangered, attempts toward a more detailed characterization of SeHV were not communicated when the work described in this thesis commenced. Chapter 2 is dedicated to the diagnosis, identification and molecular characterization of seal herpesviruses and their phylogenetic relationships to herpesviruses of dogs and cats. These studies led to the distinction of two separate seal herpesvirus species of α– and γ–herpesvirus lineage, respectively. A significant age-dependent clinical impact of PhHV infections in hospitalized seals is demonstrated. As a first step toward a vaccine confering protection against PhHV-induced disorders, major immunogenic proteins of this virus are identified, including the glycoprotein B (gB) equivalent. The gB-encoding gene has been sequenced and expressed in insect cells. Perspectives for a future subunit PhHV vaccine are evaluated.

In chapter 3, general aspects of the antigenic and phylogenetic relationships of morbilliviruses of seals, dogs and cats are studied. Furthermore, investigations are presented which unravel the molecular identity of morbilliviruses involved in recent distemper outbreaks among exotic *Felidae* in the USA and the Tanzanian Serengeti National Park.

An extensive summarizing discussion is presented in chapter 4. The main focus is on the phylogenetic relationships of morbilli- and herpesviruses of aquatic and terrestrial carnivores. Lines of future research with regard to molecular epidemiology and prevention of disease associated with these viruses are discussed.

Chapter 2

Herpesviruses of seals, dogs and cats

Section 2.1

Comparative immunological characterization of type-specific and conserved B-cell epitopes of pinniped, felid and canid herpesviruses.

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Abstract — Murine monoclonal antibodies (MAbs) were generated against phocid herpesviruses (PhHV 2557/Han88 and 7848/Han90) isolated from European harbour seals (*Phoca vitulina*), and against strain both felid (FHV strain FVR 605) and canid herpesviruses (CHV isolate 5105/Han89). MAbs were characterized with respect to certain biological properties and used to outline antigenicity profiles of isolates of PhHV (n=8), FHV (n=7) and CHV (n=3) in enzyme immunoassays employing fixed infected cells. A close antigenic relationship between herpesviruses derived from pinnipeds and terrestrial carnivores became evident: The majority of the MAbs was directed against epitopes which were expressed by at least two of the viral species tested. A number of MAbs detected epitopes which were conserved between all isolates of PhHV, FHV and CHV. A few MAbs recognized type-specific B-cell epitopes and facilitated the identification of single viral species. Moreover, the PhHV isolate 7848/Han90 was antigenically distinguishable both from seven other phocid herpesvirus isolates and from FHV or CHV. PhHV 7848/Han90 proved to be antigenically distinct from all other viruses tested when examined by cross neutralization utilizing various reconvalescent and hyperimmune sera. Although more data are needed to ensure that PhHV 7848/Han90 indeed is a new genuine seal herpesvirus, the preliminary clustering of two groups of phocid herpesvirus isolates, tentatively designated PhHV-1 (type isolate 2557/Han88) and PhHV-2 (represented by 7848/Han90), seems to be justified. By using selected MAbs an unambiguous identification and typing of herpesvirus isolates derived from marine mammals and terrestrial carnivores is significantly facilitated.

Introduction

Herpesvirus infections associated with pneumonia and focal hepatitis in hospitalized harbour seal pups *(Phoca vitulina)* have been initially described in 1985 (Osterhaus *et al.*, 1985). The so–called phocid herpesviruses (PhHV) were considered α–herpesviruses (Roizman *et al.*, 1992) with respect to their physico-chemical composition, a short replicative cycle leading to rapid mass destruction of susceptible seal kidney cell cultures, and an antigenic relationship to both felid and canid herpesviruses (FHV, CHV) detected by cross neutralization assays (Osterhaus *et al.*, 1985).

Since 1985 further herpesviruses were isolated from a California sea lion *(Zalophus californianus)* (Kennedy-Stoskopf *et al.*, 1986) and, more frequently, from harbour seals during the 1988 European morbillivirus seal mass mortality (Osterhaus, 1988; Frey *et al.*, 1989). Serosurveys indicated that infections of pinnipeds by PhHV or other closely related herpesviruses occur frequently and worldwide, including Arctic and Antarctic seal species (Vedder *et al.*, 1987; Harder *et al.*, 1991a; Stenvers *et al.*, 1992b). Firm data on the incidence and kind of clinical disease in free-ranging seal populations due to herpesvirus infections is lacking. An association with respiratory disease and abortion, however, has been suspected (Harder *et al.*, 1991a; Stenvers *et al.*, 1992b).

In the course of a natural infection, as described by Osterhaus *et al.* (1985), a mortality rate of about 50% was observed in hospitalized seal pups. Fatal, natural infections of adult seals associated with high titres of herpesvirus in central nervous tissues have been reported in cases of concomitant infections with virulent phocid distemper morbillivirus (PDV) (Zhang

et al., 1989). In contrast, experimental infection of susceptible juvenile harbour seals by the PhHV isolate 2501/Han88 resulted in an almost subclinical infection (Horvat *et al.*, 1989). Apparently, the symptomatology due to PhHV infections may be variable, depending on the seal's age, properties of the virus strain and concomitant infections, among other factors.

Evidence for an immunological relationship between PhHV and felid as well as canid herpesviruses is based on data obtained from cross neutralization experiments (Osterhaus *et al.*, 1985; Harder *et al.*, 1991a; Stenvers *et al.*, 1992b). Further attempts to characterize pinniped herpesviruses have not been reported.

Monoclonal antibodies (MAbs) proved to be excellent tools both for taxonomic and diagnostic purposes in various herpesvirus species (Cropper *et al.*, 1992; Dubuisson *et al.*, 1989; Friedli *et al.*, 1987). In order to specify the antigenic relationships among recent herpesvirus isolates of aquatic mammals and terrestrial carnivores, monoclonal antibodies were raised against two isolates of PhHV and a strain of both FHV and CHV. Here we show that the newly established panel of MAbs is useful for an unambiguous typing of isolates. Furthermore, we will provide evidence for the existence of two types of phocid herpesviruses which can be differentiated with respect to biological, immunological and epidemiological characteristics.

Materials and methods

Viruses and Cells — Isolates and strains of PhHV, FHV, CHV and SHV-1 used in this study are summarized in Tab. 1. The majority of these isolates has been described earlier (Frey, 1972; Bittle *et al.*, 1975; Povey, 1975; Borst *et al.*, 1986; Osterhaus *et al.*, 1985; Frey *et al.*, 1989; Truyen *et al.*, 1989; Harder *et al.*, 1991a). The PhHV–isolates 2010/Han89 and 7848/Han90 were isolated in primary seal kidney cultures. Isolate 2010/Han89 originated from lung tissue homogenates of a carcass of an adult seal. Cultivation of peripheral mononuclear blood cells of an adult harbour seal showing severe respiratory disease yielded isolate 7848/Han90. Both isolates, like other phocid herpesviruses, replicated in a clone of CrFK cells (Crandell *et al.*, 1973), requiring no phase of adaptation. Canine herpesviruses were isolated in primary canine kidney cell cultures: 5105/Han89 originated from lung tissues of a dog with concomitant distemper virus infection; CHV 11191/Han91 and 11361/Han91 were isolated from kidney and liver tissues, respectively, of newborn pups of two different litters which died due to generalized disease. For further propagation of CHV the canine fibroblast cell line A 72[1] was employed (Binn *et al.*, 1980). Herpesvirus isolates of feline origin were recovered by use of a feline semicontinuous cell line (cat fetus cells[2]) or CrFK cells from oropharyngeal or conjunctival swabs of cats with upper respiratory disease or conjunctivitis (Harder *et al.*, 1993a). The suid herpesvirus-1 isolate was also grown on CrFK cells. All viruses were subjected to at least three cycles of plaque purification before further use in this study.

For the preparation of concentrated virion suspensions large scale roller cultures of CrFK (PhHV, FHV) or A 72 cells (CHV) were harvested when viral cytopathic destruction was advanced, usually after three days of incubation. Following one freeze/thaw–cycle cellular debris was removed by centrifugation at 10.000 x G (15 minutes, 4°C). Clarified supernatants were subjected to ultracentrifugation for 1 hour at 85.000 x G. The pellets were resuspended in TNE-buffer (10 mM Tris-HCl,

[1]Kindly donated by Dr. S. Kölbl, Bundesanstalt für Virusseuchenbekämpfung, Vienna, Austria
[2]Supplied by Dr. K. Danner, Behringwerke AG, Marburg/Lahn, F.R.G.

pH 7.4, 100 mM NaCl, 1 mM EDTA) and centrifuged through a sucrose cushion (30% w/v in TNE) at 85.000 x G for 2 hours. Virion-enriched pellets were resuspended in TNE-buffer and repelleted at 85.000 x *G* for 40 minutes. The pellets were reconstituted in 5x volume of TNE-buffer, aliquoted in 0.2 ml portions and stored at - 80°C until use.

Monoclonal antibodies — Splenocytes obtained from Balb/c mice which had been hyperimmunized with concentrated virion suspensions emulsified in equal volumes of Freund's complete and incomplete adjuvant, respectively, were fused with cells of the X 63-AG 8.653 myeloma line (Kearney *et al.*, 1978) according to standard protocols. Hybridoma were grown in selective medium (Littlewoods *et al.*, 1968) to which as a source of cytokines supernatant of a fetal bovine splenocyte culture stimulated with Concanavalin A was added to 20% (v/v). The peroxidase-linked antibody assay (PLA) was used to identify hybridoma colonies secreting antigen-specific antibodies. Concentrates of MAbs were obtained by immune-affinity chromatography from cell culture supernatants of stabile producing hydridoma lines which had undergone at least two cycles of single-cell cloning by limiting dilution.

Peroxidase-linked antibody assay (PLA) — The PLA technique, an enzyme-linked immuno assay employing heat-fixed infected cells, was adopted from Harder *et al.* (1991a). Antibody-containing solutions were applied to heat-fixed cultures in microtitre plates and incubated for two hours at room temperature or overnight at 4°C. For the initial screening of hybridoma colonies, supernatant was added in a single dilution of 1/2 in PBST. Bound antibodies were detected by using horseradish-peroxidase (HRPO)-labeled caprine-anti-murine-IgG, and 3-amino-9-ethylcarbazole serving as the chromogen. The highest dilution of a MAb solution readily enabling signal detection by light microscopy was recorded as its PLA-titre.

For photografic documentation of the intracellular herpesviral antigen accumula high contrast immunocytochemical staining was achieved by use of 3,3'- diaminobenzidine and subsequent gold/silver enhancement according to Lazar & Taub (1992). Hematoxylin was used for counterstaining (Morris, 1990).

Plaque reduction neutralization assay (PRNT) — MAb concentrates were diluted to a protein concentration of approximately 25 - 50 $\mu g\ ml^{-1}$ in DMEM to be tested for neutralizing capacities against 50 - 100 plaque forming units (pfu) of their homologous virus (PhHV 2557/Han88, PhHV 7848/Han90, FHV FVR 605 and CHV 5105/Han89). A polyclonal serum known to neutralize the respective virus in a microneutraliz assay (see below) served as positive control. A neutralizing MAb raised against the H-protein of phocid distemper virus (PDV) represented the negative control (Harder *et al.*, 1991a). Virus-MAb or -polyclonal serum mixtures were incubated overnight at 4°C before inoculation in duplicate onto monolayers of susceptible (either CrFK or A 72) cells for an adsorption period of 2 hours. In a parallel series, virus-MAb mixtures were incubated in the presence of 4% (v/v, final) of ginuea pig complement. After incubation for 72 hours plaques formed by PhHV 2557/Han88, FHV FVR 605, and CHV 5105/Han89 were macroscopically visible. In the case of PhHV 7848/Han90 the incubation period was prolonged to 6 days and a second overlay was added at day 4. Plaques were then immunocytochemically stained by the PLA-technique utilizing MAb 7848 2.4D7 or 6E5 as the first layer.

The percentage reduction of plaque formation by a certain MAb was calculated with respect to the number of plaques induced in the negative control.

Table 1. Origin of herpesviruses.

Virus	Host	Disease	Specimen	Reference
PhHV				
PB 84	*Ph. vitulina*	respiratory	kidney culture	Osterhaus *et al.* (1985)
PB 6–II/85	*Ph. vitulina*	respiratory	lung	Osterhaus *et al.* (1985)
2158/Han88	*Ph. vitulina*	respiratory	lung	Frey *et al.* (1989)
2160/Han88	*Ph. vitulina*	respiratory	liver	Frey *et al.* (1989)
2501/Han88	*Ph. vitulina*	respiratory	lung	Frey *et al.* (1989)
2557/Han88	*Ph. vitulina*	respiratory	leucocytes	Frey *et al.* (1989)
2010/Han89	*Ph. vitulina*	carcass	lung	this work
7848/Han90	*Ph. vitulina*	generalized	leucocytes	this work
FHV				
FVR 605	*Catus felis*	–	vaccine strain[1]	Povey (1975)
F 2	*Catus felis*	–	vaccine strain[2]	Bittle *et al.* (1975)
FHV/L	*Panthera leo*	encephalitis	tonsil	Truyen *et al.* (1989)
6084/Han90	*Catus felis*	rhinotracheitis	pharyng. swab	Harder *et al.* (1993a)
6887/Han91	*Catus felis*	rhinotracheitis	pharyng. swab	Harder *et al.* (1993a)
299/Han91	*Catus felis*	conjunctivitis	conjunct. swab	Harder *et al.* (1993a)
10499/Han91	*Catus felis*	rhinotracheitis	pharyng. swab	Harder *et al.* (1993a)
CHV				
5105/Han89	*Canis familiaris*	respiratory	lung	Harder *et al.* (1993a)
11190/Han91	*Canis familiaris*[3]	generalized	kidney	this work
11361/Han91	*Canis familiaris*[3]	generalized	liver	this work
SHV-1				
48-1/Han68	*Sus domesticus*	respiratory	lung	Frey (1972)

[1] Dr. K. Danner, Behringwerke AG, Marburg/Lahn, F.R.G.; [2] Dr. A. Aubert, Virbac Laboratories, Carosse, France; [3] Newborn whelps.

Microneutralization assay (MNT) — Sera of naturally and experimentally herpesvirus-infected animals were assayed for their neutralizing capacities in the presence of 4% (v/v) guinea pig complement solution against PhHV, FHV, CHV and SHV-1. The test was essentially performed as described (Harder *et al.*, 1991a). 50% end point titres were calculated according to Kaerber (1931).

Results

Growth properties of phocid herpesviruses *in vitro*

All PhHV isolates replicated in primary seal kidney cells as well as in CrFK cells, but not in cells of canine origin. However, neither FHV nor CHV could be adapted to phocine cells by three blind passages (data not shown). Cytopathogenic effects induced by all PhHV isolates

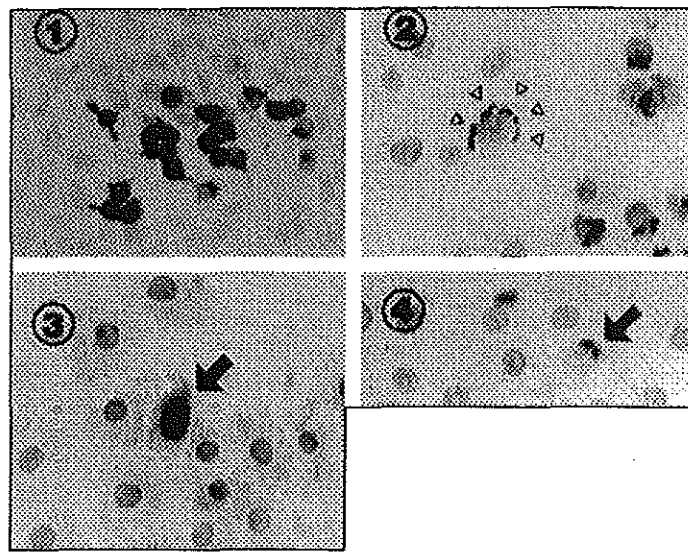

Figure 1. Intracellular distribution of antigen in CrFK cells infected by phocid herpesvirus isolates identified by high contrast peroxidase-linked immunocytochemical staining utilizing different monoclonal antibodies. 1. Plaque of CrFK cells infected by PhHV-1 2557/Han88 and pancytoplasmically stained using MAb PhHV-1 2557 3.1D3. 2. Perinuclear localization of antigen (open triangles) identified by MAb PhHV-2 7848 1.6C5 in CrFK cells infected by PhHV-2 7848/Han90. 3. Pannuclear distribution pattern of antigen stained by MAb PhHV-2 7848 1.9A5 in CrFK cells infected by PhHV-2 7848/Han90. 4. Polar accumulation of antigen (closed arrow) in a nucleus of a CrFK cell infected by PhHV-2 7848/Han90 (MAb PhHV-2 7848 1.7C8).

except 7848/Han90 were characterized by development of single rounded single cells gradually detaching from the vessel surface (Fig. 1). The onset of cytopathogenic effects induced by PhHV 7848/Han90 was delayed in comparison to other PhHV isolates and consisted mainly of syncytia formation. Under a semisolid overlay PhHV 2557/Han88, FHV and CHV strains generated macroscopically visible plaques of 1 - 3 mm in diameter within three days. Plaques induced by PhHV 7848/Han90, in contrast, did not exceed pin-point size even after prolonged incubation of six days.

Accumulation sites of viral antigens in CrFK cells infected by PhHV

A high-contrast peroxidase-linked immunocytochemical staining assay was used to demonstrate the accumulation of PhHV viral antigens in infected cells (Fig. 1). Using the panel of MAbs raised against the PhHV isolates 2557/Han88 and 7848/Han90 three different patterns were identified (Tab. 2):

Table 2. Characterization of MAbs raised against phocid herpesvirus isolates 2557/Han88 and 7848/Han90, felid herpesvirus strain FVR 605 and canid herpesvirus isolate 5105/Han89.

MAb designation	Antigen localization[1]	Neutralization[2]
PhHV 2557/Han88[3]		
1.1H3*, 2.2F2*	cytoplasmic	100
3.4B3, 3.4G1, 3.4G6, 3.5F1, 3.6A12, 3.6E7, 3.7B9	cytoplasmic	60 - 90
2.3F4, 2.3F7, 2.5B4, 2.9A12, 3.1D3, 3.3G8, 3.3H5, 3.5B9, 3.7G3	cytoplasmic	$\leq$ 20
3.2C8, 3.3E4	nuclear	$\leq$ 20
PhHV 7848/Han90[3]		
1.3E5, 2.4D7, 2.6E5	cytoplasmic	$\leq$ 20
1.4C6, 1.6C5	perinuclear	$\leq$ 20
1.7C8, 1.8C3, 1.9A5	nuclear	$\leq$ 20
FHV FVR 605[3]		
1.4A1, 1.5H10, 1.8H6	cytoplasmic	$\leq$ 20
CHV 5105/Han89[3]		
2.3A10	cytoplasmic	70
1.2G7, 1.5A2, 2.9F6	cytoplasmic	$\leq$ 20

[1] Tested by peroxidase-linked antibody assay.

[2] Percentage reduction of plaque forming units of the homologous virus (50 pfu) by 25-50 μg of antibody in the presence of 4% (v/v) of guinea pig complement.

[3] Virus used to raise the MAbs; * MAbs did not require complement for neutralization.

The majority of the MAbs stained viral antigen which was evenly distributed throughout the cytoplasm (Fig. 1.1). Antigens detected by MAbs 7848 1.4C6 and 1.6C5 showed a restricted perinuclear localization surrounding the nucleus like a corona. In early stages of viral replication this antigen was found in the cytoplasm near the poles of the nucleus (Fig. 1.2). Some MAbs recognized viral antigen which was confined to the nucleus (Figs. 1.3, 1.4). While nuclei of cells apparantly in early stages of viral replication revealed a scattered staining pattern (Fig. 1.4), enlarged nuclei of cells located in the centre of viral plaques were homogeneously filled with antigen (Fig. 1.3).

Identification of conserved and type-specific epitopes of phocid, felid and canid herpesviruses

The reactivity and affinity of MAbs established against different herpesviruses was determined by use of an enzyme immunoassay employing fixed infected cells (peroxidase-linked antibody assay, PLA). As a measure of relative antibody affinity (AF-index) the ratio between the PLA-titre and the protein content (IgG) ml^{-1} of a given MAb concentrate was calculated.

As shown in Fig. 2 out of 20 MAbs raised against PhHV 2557/Han88 all but one reacted with CHV isolates whereas nine had also a high affinity to most FHV strains. Three MAbs, namely 2557 2.3F4, 2.3F7 and 2.9A12, also weakly reacted with pseudorabies virus (data not shown). The PhHV isolate 7848/Han90 was distinguishable both from seven other phocid herpesvirus isolates as well as from CHV and FHV (Fig. 2): Only four of the 20 MAbs produced against PhHV 2557/Han88 also detected PhHV 7848/Han90 antigen. In contrast, the majority of MAbs raised against the latter virus recognized other PhHV isolates but displayed only limited reactivity with FHV and CHV. Most of the MAbs produced against FHV and CHV reacted with all PhHV isolates except PhHV 7848/Han90.

The monoclonal antibodies 2557 3.6E7; 7848 1.4C6, 1.6C5, 1.7C8, 2.4D7; FVR 2.8H6 and CHV 2.9F6 were found to bind antigen of other except their homologous virus species with very low affinity, if at all. Thus, these MAbs were used for identification of single viral species. In combination with cross reactive MAbs like 2557 3.3G8 and 3.1D3 an unambiguous antigenic identification and typing of herpesvirus isolates derived from terrestrial carnivores and pinnipeds using the PLA technique can easily be accomplished.

In general, isolates of PhHV (except 7848/Han90) and CHV displayed homogeneous reactivity patterns to the MAb panel. However, among felid herpesviruses, a diffuse intraspecies antigenic heterogeneity was detectable with some MAbs: MAb 5105 1.2G7, for example, moderately reacted with FHV/L, FHV 6084, and 6887 but not with other FHV isolates including the vaccine strains. A general and concise clustering, e.g. into vaccine strains and isolates obtained from cats showing signs of rhinotracheitis or conjunctivitis, was not possible.

Neutralization of viral infectivity by monoclonal antibodies

A number of MAbs generated against PhHV 2557/Han88, FHV and CHV partially (40 - 80%) reduced the formation of plaques of their homologous virus when assayed in a comparatively high protein concentration of 25 - 50 μg ml^{-1} (Tab. 2). Only two MAbs generated against PhHV 2557/Han88 (1.1H3 and 3.2F2) completely neutralized viral infectivity even without complement supplementation and in higher dilutions. However, these MAbs were uncapable of cross neutralizing other herpesvirus species (data not shown).

Differential neutralization by sera

A complement-enhanced microneutralization assay was used to investigate whether PhHV 7848/Han90 can also be distinguished f PhHV isolates by polyclonal antibodies derived from various naturally and experimentally herpesvirus-infected species. For use in this study pinniped sera were selected which displayed high neutralizing titres to the PhHV isolate 2557/Han88. The majority of these sera also strongly neutralized CHV (Tab. 3). In addition, sera sampled from European harbour seals cross neutralized FHV whereas this was not achieved with sera of Antarctic Weddell seals. All sera, however, enabled the differentiation of the phocid herpesvirus isolate 7848/Han90 which appeared to be more distantly related to PhHV 2557/Han88 than FHV but not as distant as SHV-1.

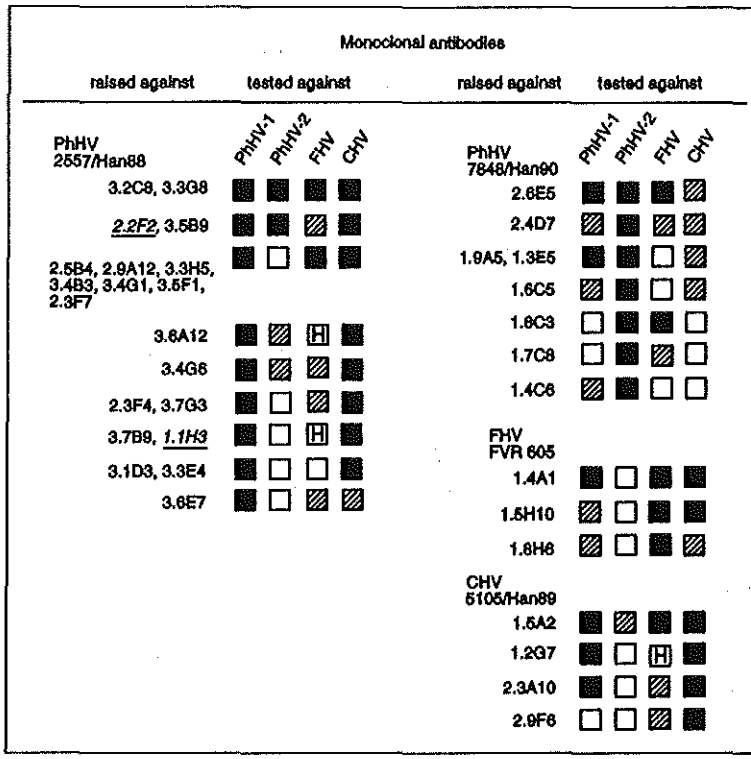

Figure 2. Reactivity of monoclonal antibodies (MAbs) against phocid (PhHV), felid (FHV) and canid (CHV) herpesviruses analyzed by peroxidase-linked antibody assay. Reactivity gradations are based on affinity values (see material and methods for calculation): ■ strong, ▨ weak, H – heterogeneous, □ not detectable. With respect to the reactivity patterns the herpesvirus isolates and strains tested could be clustered as follows: PhHV-1 (n=7): PB 84, PB 6-II/85, 2158/Han88, 2160/Han88, 2501/Han88, 2557/Han88, 2010/Han89; PhHV-2 (n=1): 7848/Han90; FHV (n=7): FVR 605, F2, FHV/L, 6084/Han90, 6887/Han91, 299/Han91, 10499/Han91; CHV (n=3): 5105/Han89, 11190/Han91, 11361/Han91. Underlined letters indicate single MAbs which were shown to completely neutralize their homologous virus independently from complement supplementation.

Discussion

Previous studies based on cross neutralization experiments employing reconvalescent seal sera have demonstrated an antigenic relationship between genuine herpesviruses isolated from cats, dogs and European harbour seals (*Phoca vitulina*) (Osterhaus *et al.*, 1985; Harder *et al.*, 1991a ; Stenvers *et al.*, 1992b). More detailed comparisons of FHV and CHV led to the identification of some glycoproteins sharing a number of conserved epitopes (Limcumpao *et al.*, 1990; Rota & Maes, 1990).

Table 3. Cross neutralization of phocid herpesviruses and antigenically related herpesviruses of terrestrial carnivores.

Serum	PhHV-1 2557/Han88	PhHV-2 7848/Han90	FHV FVR 605	CHV 5105/Han89	SHV-1 48/Han68
Weddell seals					
5255/91	65[1]	≤ 5	15	53	≤ 5
5257/91	40	≤ 5	≤ 5	8	≤ 5
5258/91	52	≤ 5	≤ 5	53	10
5259/91	40	≤ 5	≤ 5	53	≤ 5
5272/91	52	≤ 5	≤ 5	28	≤ 5
Harbour seals					
3128/88	220	40	80	46	7
3394/88	80	≤ 5	28	130	≤ 5
3400/88	80	≤ 5	92	113	≤ 5
7848/90	450	46	46	180	≤ 5
8549/92	48	≤ 5	≤ 5	≤ 5	≤ 5
11135/92	450	≤ 5	130	130	≤ 5
11142/92	370	≤ 5	46	23	≤ 5
11164/92	192	26	23	32	≤ 5
11166/92	256	≤ 5	160	32	≤ 5
11173/92	450	105	23	56	≤ 5
Reference sera					
725C	13	≤ 5	≤ 5	40	≤ 5
αFVR605S	14	≤ 5	3100	≤ 5	≤ 5
AK-F	≤ 5	≤ 5	≤ 5	≤ 5	92

[1] Reciprocals of ND_{50}.

Sera nos. 5255 to 5272 were collected from Antarctic Weddell seals (*Leptonychaetes weddellii*) in 1990 (Harder *et al.* 1991a). Nos. 3128 to 11173 originated from free-ranging harbour seals (*Phoca vitulina*) of the German wadden sea 1988 - 1992. Serum 725C resulted from a dog experimentally infected by CHV 5101/Han89 (Harder 1990, unpublished). The anti-FHV serum (αFVR605S) was raised in a sheep against FVR 605 (Dr. K. Danner, Behringwerke AG, Marburg, F.R.G.). A porcine field serum served as a reference for SHV-1-specific antibodies.

By using a new panel of monoclonal antibodies raised against two PhHV isolates we confirm and extend these data. Eight phocid herpesvirus isolates obtained during 1984 - 1990 from different harbour seals with different clinical syndromes were taken under study and compared to vaccine strains and recent field isolates of felid and canid herpesviruses. Seven PhHV isolates displayed a homogeneous pattern of reactivity to 35 MAbs. Isolates of seemingly different pathogenicity (e.g. PhHV PB 84 and 2501/Han88) could not be distinguished. The isolate 7848/Han90, in contrast, differed in a number of epitopes defined by MAbs from other PhHV isolates as well as from FHV and CHV. Multiple cross neutralization experiments

employing reconvalescent sera of different seal species as well as reference sera to FHV or CHV and a serum from a SHV-1 infected pig gave further evidence that PhHV 7848/Han90 is immunologically distinct, though distantly related, from all other viruses tested here. In addition, some *in vitro* properties, e.g. comparatively slow growth with predominant formation of syncytia, are not shared with other PhHV isolates. 7848/Han90 was isolated from leucocytes, but not from other tissues of an adult harbour seal which succumbed to a respiratory disease. The isolation of herpesviruses from seals sharing the properties of 7848/Han90 had not yet been reported. Definite proof that 7848/Han90 indeed represents a new genuine seal herpesvirus thereby ruling out the possibility of a contamination, e.g. originating from fetal calf serum, must come from restriction enzyme analysis and sequence data of the genome. Until then the differentiation of two distinct groups of phocid herpesviruses and their tentative designation as PhHV-1 (represented by PhHV 2557/Han88) and PhHV-2 (represented by PhHV 7848/Han90) may be justified.

Some diffuse and heterogeneous antigenic differences were also observed among vaccine strains of FHV and herpesvirus isolates recovered from diseased cats. However, a correlation of loss or presence of single antibody binding sites and clinical entities was not feasible.

Isolates of the proposed PhHV-1 type displayed a pronounced cross relationship especially to CHV and to a lesser extent also to FHV. A similar situation was unraveled by cross neutralization experiments. Titres of neutralizing antibodies in sera derived from different pinniped species were generally highest against PhHV-1 (2557/Han88). The majority of sera, at least those derived from European harbour seals, significantly cross neutralized CHV 5105 and FHV FVR 605 in contrast to PhHV-2 (7848/Han90). The serum of the seal from which PhHV-2 7848/Han90 was isolated displayed a high titre to PhHV-2. As an unexpected finding the titre of this serum against PhHV-1 was even higher. For an explanation it may be assumpted that seal 7848 has previously been infected by PhHV-1; upon a recent primary infection with PhHV-2 (7848/Han90) a presumably latent PhHV-1 infection was reactivated leading to a boost or an anamnestic response of PhHV-1 specific antibodies while PhHV-2-specific antibodies yet were not fully developed. However, it can not be excluded that infections with PhHV-2 are not inducing neutralizing antibodies as readily as PhHV-1. Serological follow-up studies in this case were not possible since the animal died within a few days.

The host restriction of many α-herpesviruses both *in vivo* and *in vitro* is variable (Roizman *et al.*, 1992). Some viruses, e.g. CHV, appear to be strictly confined to a single host species while others such as pseudorabies virus are capable of crossing host species boundaries. Diseases induced by most α-herpesviruses in their natural hosts are commonly self-limiting. Aberrant host species, in contrast, are more prone to develop severe and often fatal clinical syndromes. Herpesviruses were isolated from the faeces of dogs showing signs of gastroenteritis. These isolates replicated in feline cell cultures and were indistinguishable from felid herpesvirus by cross neutralization (Everman *et al.*, 1982), restriction enzyme analysis and Southern blotting (Rota *et al.*, 1986). The infection of dogs with a felid herpesvirus variant provoked a clinical syndrome which is generally not seen in FHV infections of the natural host (Povey, 1979). Data whether PhHV is carrying any potential to cross the species boun-

daries and infects members of the *Canidae* or *Felidae* families are presently not available. It is tempting to speculate that the latter would not be unlikely since both proposed types of PhHV did not require an adaptation phase to replicate productively in feline cell cultures. In contrast, cells of canine origin seem to be non-permissive. A system enabling an unambiguous identification and differentiation of herpesviruses isolated from sea mammals and terrestrial carnivores would be desirable both for diagnostic and epidemiological reasons. For this purpose some of the newly established MAbs are suitable (PhHV-1: 2557 3.6E7, PhHV-2: 7848 1.4C6, FHV: FVR 8H6, CHV: 5105 2.9F6). Once an isolate is available it can be typed by PLA within a couple of hours avoiding the more time consuming, laborious and sophisticated restriction enzyme analysis.

The panel of herpesvirus-specific MAbs characterized here may also aid in the diagnosis of herpesvirus infections in cetaceans which have recently been described in a harbour porpoise (*Phocoena phocoena*) showing encephalitis (Kennedy *et al.*, 1992) and in Beluga whales (*Delphinapterus leucas*) with dermatitis (Martineau *et al.*, 1988; Barr *et al.*, 1989).

In conclusion, using a large panel of MAbs we provided evidence that herpesviruses of harbour seals are antigenically related to, though distinct from felid and canid herpesvirus. Two groups of phocid herpesviruses, tentatively designated PhHV-1 and PhHV-2 were differentiated by cross neutralization experiments and epitope-typing using the newly established MAb panel. While only a few antigenic differences between PhHV-1, CHV and FHV were detected PhHV-2 appears to be more distantly related to PhHV-1 isolates. Further work is in progress to characterize the genomes and proteins specified by PhHV-1 and -2 in order to confirm their classification as separate genuine pinniped herpesviruses.

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Section 2.2

Characterization of phocid herpesviruses-1 and -2 as putative alpha- and gamma-herpesviruses of North American and European pinnipeds

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Abstract — To study the relationships between herpesviruses recently isolated from different pinniped species, antigenic and genetic analyses were performed. First, herpesviruses isolated from North American harbour seals (*Phoca vitulina*), a California sea lion (*Zalophus californianus*) and a European grey seal (*Halichoerus grypus*) were examined in an enzyme immunoassay (EIA) with a panel of monoclonal antibodies which had previously been shown to allow typing of herpesviruses from European harbour seals into two distinct virus types: phocid herpesvirus type-1 and type-2 (PhHV-1 and PhHV-2). The EIA data showed all but one of the isolates from seals ranging in United States coastal waters were PhHV-2-like while the European grey seal herpesvirus was PhHV-1-like. Genetic characterization was facilitated by PCR analysis using primers based on conserved regions of the glycoprotein B and D (gB and gD) genes of the antigenically closely related canid (CHV) and felid (FHV) herpesviruses. Specific amplified products were obtained with five isolates antigenically characterized as PhHV-1-like but not with five PhHV-2-like isolates. Sequence analysis of the PCR products confirmed greatest similarity to members of the genus *Varicellovirus* of the α-*Herpesvirinae* and in particular to CHV. Sequence analysis of two *Eco*RI fragments of the PhHV-2 genome (European isolate 7848) revealed greatest similarity to γ-herpesviruses and in particular equine herpesvirus-2. Although an unambiguous subgrouping was not feasible, this is the first evidence that PhHV-2 may be a putative γ-herpesvirus of pinnipeds.

Introduction

Herpesviruses which are antigenically closely related to felid and canid herpesvirus (FHV, CHV) have been isolated from pinnipeds (Osterhaus *et al.*, 1985; Kennedy-Stoskopf *et al.*, 1986; Osterhaus & Vedder, 1988; Frey *et al.*, 1989; Horvat *et al.*, 1989; Lebich *et al.*, 1994). Herpesvirus isolates obtained from European harbour seals (*Phoca vitulina*) are currently referred to as phocid herpesvirus (PhHV) and were tentatively classified as belonging to the subfamily α-*Herpesvirinae* (Osterhaus *et al.*, 1985; Roizman *et al.*, 1992). The clinical relevance of herpesvirus infections in free-ranging pinniped populations is still poorly understood. However, in animals in captivity, severe outbreaks of herpesvirus infections have occurred accounting for mortality rates of up to 50% among seal pups due to severe pneumonia and generalization of the herpesvirus infection (Osterhaus *et al.*, 1985).

The immunological relationships between European PhHV isolates and herpesviruses of terrestrial carnivores have been investigated with panels of monoclonal antibodies (MAbs) and with convalescent seal sera. These studies provided evidence for the existence of at least two different herpesviruses (PhHV-1, PhHV-2) in European harbour seal populations (Lebich *et al.*, 1994). Isolates of the PhHV-1 type were antigenically closely related to, though distinct from CHV and FHV. PhHV-2, as yet represented only by a single isolate from a harbour seal of the German Wadden Sea, proved to be more distantly related to PhHV-1 and the herpesviruses of cats and dogs. Serological surveys indicated that herpesviruses which are closely related to European PhHV-1 are also present in seal populations of Antarctica and the Northern Pacific (Vedder *et al.*, 1987; Harder *et al.*, 1991a; Stenvers *et al.*, 1992b).

Here we present the data from a comparative analysis of herpesviruses recently isolated from North American and European pinniped species, obtained using a panel of MAbs, and

sequence data obtained by amplification of fragments of the gD and gB genes of PhHV-1-like viruses. In addition, sequences of *Eco*RI fragments of the PhHV-1 genome are presented.

Materials and methods

Virus and cells – Plaque-purified reference isolates of PhHV-1 (PB84, 2557/Han88) PhHV-2 (7848/Han90) and FHV (6887/Han90) were propagated in Crandell feline kidney cells (CrFK) while the CHV isolate 5105/Han89 was grown in Madin-Darby canine kidney (MDCK) cells as described (Lebich *et al.*, 1994). Seal kidney cell cultures (SeKC) were generated and maintained as described (Osterhaus *et al.*, 1985). Vero cells used to multiply two previously undescribed phocid herpesvirus isolates (A93-2/2 and A93-2/11) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum (FCS). The origin and cultural properties of newly isolated pinniped herpesviruses are summarized in Table 1.

Replication kinetics of PhHV-1 and -2 in CrFK cells – CrFK cells were seeded at 2 x 10^5 cells in 2 ml of medium into roller tubes and incubated stationary overnight. Medium was withdrawn and cultures were inoculated with PhHV-1 2557/Han88 or PhHV-2 7848/Han90 at an m.o.i. of 1. Adsorption was allowed to occur for two hours at 37°C while rolling. Then cultures were washed twice with PBS and reconstituted with 1 ml medium for further stationary incubation at 37°C. At set intervals two tubes each were withdrawn. Extracellular virus yield was measured in the pooled cell-free supernatants. Debris and cells were reconstituted in 1 ml of fresh cell culture medium and frozen at - 80°C. After defrosting and removal of cellular debris the cell-bound viral infectivity was titrated. Infectivity was measured in a microtitration assay based on the development of cytopathic changes (Frey *et al.*, 1989). Titrations of PhHV-1 were incubated for 4 days while those of PhHV-2 required up to 10 days before cytopathic changes had developed sufficiently.

Immunological characterization of virus isolates – A selection of MAbs from a panel previously raised against European PhHV-1 and -2 isolates, FHV and CHV (8) was used to characterize viral antigens in an enzyme immunoassay using heat-fixed infected cells as described (Lebich *et al.*, 1994), except that instead of PBS containing Tween 20 double-distilled water was used in all washing steps.

Preparation of DNA for PCR – Herpesvirus-infected cell cultures showing advanced stages of viral cytopathic destruction were washed once with cold PBS; cells were then pelleted and lysed in 2 x vol. of a buffer containing 50 mM Tris (pH 8.0), 5 mM EDTA and 0.5% (v/v) Nonidet P-40 while incubating for 2 minutes on ice. Insoluble residues were pelleted and *N*-laurylsarcosinate (NLS, 0.5% [w/v] final concn) as well as Proteinase K (250 μg ml^{-1} final concn) were added to the supernatant. Proteinase K digestion was allowed to proceed for 2 h at 45°C. Nucleic acids were recovered by ethanol precipitation after two phenol-chloroform extractions and reconstituted in 50 μl of double-distilled water of which 5 μl were used for PCR. Uninfected cell cultures (CrFK and Vero) were treated identically.

Primer selection and polymerase chain amplification – Since nucleotide sequences of seal herpesviruses have not been reported yet, primers were selected from sequences of CHV and FHV which had been found to be the closest relatives of PhHV-1 at the antigenic level (Lebich *et al.*, 1994). With the available limited sequence data of these viruses (Maeda *et al.*, 1992, 1994; Spatz

Table 2. Partially degenerate PCR primers based on gB and gD gene equivalent sequences of canid, felid and equine (type 1) herpesviruses.

Designation	Sequence (5' – 3)	Location (CHV)	Product sizes (bp) CHV	FHV
gD-1	gAA gTT Cgg TAT gT[A/T] AC	142 → 158		
gD-2	AAT CCC AAT TCA TC[A/g] TC	415 ← 431	290	287
gB-1	ACA ACT gTA Tgg TCT gg	358 → 374		
gB-2	CAC Cgg Tgg AgA TAg C	709 ← 724	367	367
			1616	1632
gB-3	gCT ACA gTT TgC CTA TgA	1431 → 1448		
gB-4	ggT AgA AAT TCA CgA TC[C/T] TC	1954 ← 1973	543	540

* Primers were basically choosen from the CHV sequence. Nucleotides marked by a dot were selected from the corresponding sequences of FHV, whereas an asterisk marks nucleotides incorporated from EHV-1 sequences (Whalley *et al.*, 1991).
+ Data according to Limbach *et al.*, 1994; position 1: translation initiation ATg.
++ Data according to Maeda *et al.*, 1992, 1994; Spatz and Maes, 1993; Spatz *et al.*, 1994; position 1: translation initiation ATg.

& Maes, 1993; Limbach *et al.*, 1994; Spatz *et al.*, 1994), reasonably well-conserved regions were pinpointed in genes encoding the glycoprotein equivalents B and D (Table 2). Primers were made degenerate at certain positions, also incorporating nucleotides of corresponding sequences of equine herpesvirus type-1 (Whalley *et al.*, 1991). DNA-PCR (Ampli*Taq* polymerase, GenAmp 9600: Perkin-Elmer) was performed following an initial denaturation step at 94°C for 3 minutes and consisted of 33 cycles of 30" at 94°C, 1' at 37, 45 or 55°C and 1' at 72°C. A final elongation step at 72°C was allowed for 10 minutes. The PCR mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 % (v/v) Triton X-100, 200 μM of each dNTP, 0.5 nM of sense and antisense primer and 2.5 U Ampli*Taq* polymerase. $MgCl_2$ was added separately from a stock to give the desired concentration. A 5 μl volume of DNA prepared from tissue cultures was added while 20 μl of DNA solutions obtained from clinical specimens were added to give final volumes of 100 μl. Primer specificities were checked and PCR conditions optimized using DNA templates extracted from cell cultures infected with either CHV, FHV, PhHV-1 PB84 or PhHV-2 7848/Han90. Annealing temperatures of 37, 45 and 55°C were evaluated as well as Mg^{2+} concentrations of 1, 2, 3 and 4 mM. Other viral isolates (see Table 1) were subsequently assayed using the conditions found to be optimal with the above templates. Products were separated in agarose gels and visualized after ethidium bromide staining. Measures minimizing the risk of carry-over contaminations consisted of physical separation of pre- and post-PCR procedures, preparation of templates in a laminar flow cabinet, use of master mixes and aerosol-resistant tips. Negative controls (double-distilled water or medium) were routinely run in parallel.

Cloning and partial sequencing of gB and gD gene equivalent PCR products – Products obtained from PCRs using gD specific primers with template DNA of four PhHV-1 isolates (PB84, 550/Hel93, Ja/PB94, A92-10/4) were cloned into a ßtickyT plasmid vector (pCR II, InVitrogen).

Plasmids containing inserts of the correct size were selected and cycle-sequenced using T7 and M13 reverse primers in a DyeDeoxy terminator reaction (PRISM, Applied Biosystems). Samples were analysed on an automatic sequencer (model 373A, Applied Biosystems). Similarly, the 1.6 kB gB specific amplificate of PhHV-1 PB84 was cloned and partially sequenced from the 5'-end. Three clones of each amplificate were sequenced in order to correct for possible reading errors of the Ampli*Taq* Polymerase. Further sequence analysis was done with the Genetics Computer Group (GCG) software package (release 8.0).

Southern blotting – PCR amplificates were separated in 0.7% agarose gels and transferred by capillary blotting onto positively charged nylon membranes by standard procedures. Non-radioactive hybridizations were carried out using an enhanced chemoluminiscence assay (ECL, Amersham); gB or gD specific PCR products reamplified from cloned PhHV-1 PB84 PCR fragments were used as probes. Hybridization was for 4 h at 42°C. Subsequent washing steps were performed under low to moderate stringent conditions according to the manufacturer's recommendations.

Cloning and sequencing of EcoRI fragments of PhHV-2 – Virions of the PhHV-2 isolate 7848 were purified as described by Lebich *et al.* (1994). DNA was extracted following exactly the protocol of Engels *et al.* (1983). DNA was digested with *Eco*RI and fragments were cloned into pUC18. Specific clones were identified by non-radioactive colony blotting (ECL, Amersham) using full-length virion DNA as a probe. Two clones carrying PhHV-2-specific inserts of 315 and 788 bp were sequenced in completion as described above.

Results

Biological properties and antigenic relationships of newly isolated pinniped herpesviruses from the United States Pacific and Atlantic coasts as compared to European PhHV-1 and PhHV-2

Replication kinetics of two reference isolates of PhHV-1 and -2 in CrFK cells were examined. Although the experimental design did not allow for single-step growth curves (the m.o.i. for both PhHV types was 1), a more rapid replication of the PhHV-1 was evident (not shown). Virus replication was paralleled by focal appearance of rounded cells followed by the complete destruction of cell monolayers. In contrast, the infectivity of the PhHV-2 isolate remained mainly cell-associated and the onset of the viral cytopathic effect, characterized by the development of round cells and the formation of large syncytia, was markedly slower. Titers of cell associated infectivity peaked at about $10^{5.0}$ $TCID_{50}$ ml^{-1} for both isolates.

Typing with MAbs allowed a clustering of previously undescribed pinniped herpesviruses (Fig. 1). All but one of the American harbour seal isolates and the California sea lion herpesvirus (Zalo/Cal86) were classified as PhHV-2. A single MAb (PhHV-2 1.3E5) raised against the European PhHV-2 isolate 7848/Han90 discriminated between American and European PhHV-2-like isolates. All of the recent herpesvirus isolates from European seals and one isolate from an American harbour seal belonged to the PhHV-1 cluster. Minor antigenic differences were also evident within this cluster. However, the American PhHV-1 isolate was not distinguishable from the European isolates.

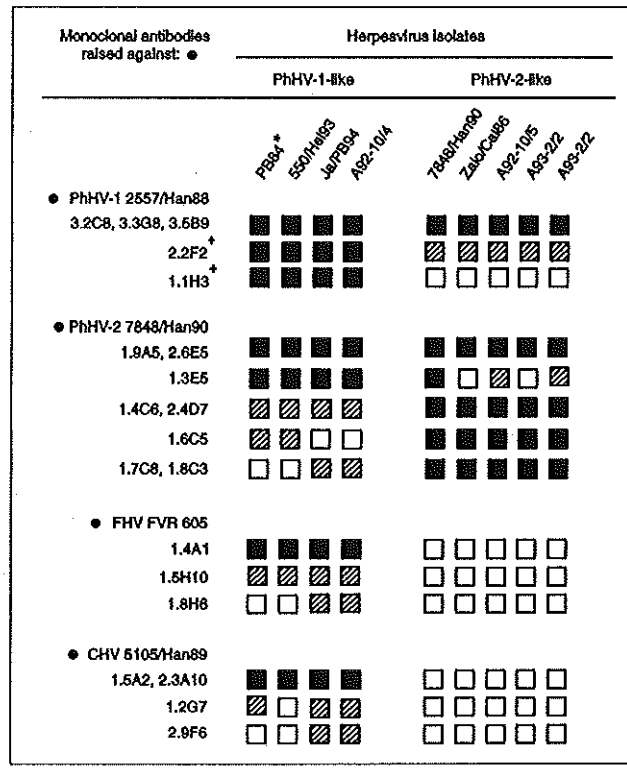


Figure 1. Reactivities of MAbs raised against various pinniped herpesvirus isolates analysed by an enzyme immunoassay employing fixed infected CrFK cells. Cross reactivity gradations were evaluated using arithmetic mean values of at least three titration experiments: Strong (■), weak (▨) and not detectable (□)) (titer $\leq$ 1:10). The differences in titer discriminating strong and weak reactions were at least one log_{10}-step. * The PhHV-1 isolate 2557/Han88 showed the same pattern. + MAbs neutralized PhHV-1 isolates in a complement-enhanced neutralization assay (Lebich *et al.*, 1994).

Amplification, cloning and partial sequencing of a 1.6 kB gB equivalent gene fragment of PhHV-1

Primer specificities in PCR were determined with template DNA prepared from infected cell cultures. Suitable conditions for the amplification of a 1.6 kB fragment using partially degenerate primers gB_1 and gB_4 from FHV, CHV and PhHV-1 PB84 DNA templates were an annealing temperature of 45°C and a 1 mM concentration of Mg^{2+}. PCR using these conditions was attempted with template DNA of all other PhHV isolates. However, amplification of a fragment of the expected size which hybridized with the cloned gB specific fragment of PhHV-1 PB84 was achieved only with CHV, FHV and pinniped herpesvirus isolates antigenically typed as PhHV-1 (Fig. 2a). Similarly, no specific amplificates were obtained with

template DNA extracted from PhHV-2 infected cell cultures using the primer pairs gB_1 – gB_2 or gB_3 – gB_4, respectively (data not shown). The PhHV-1 PB84 1.6 kB gB specific fragment was cloned and partially sequenced from its 5'-end. Alignments of a stretch of 500 nucleotides (excluding primer sequences) to gB genes of FHV and CHV confirmed the specificity of the amplificate (Fig. 3A). Nucleotide identities of 79% and 75% to CHV and FHV, respectively, were observed in that region.

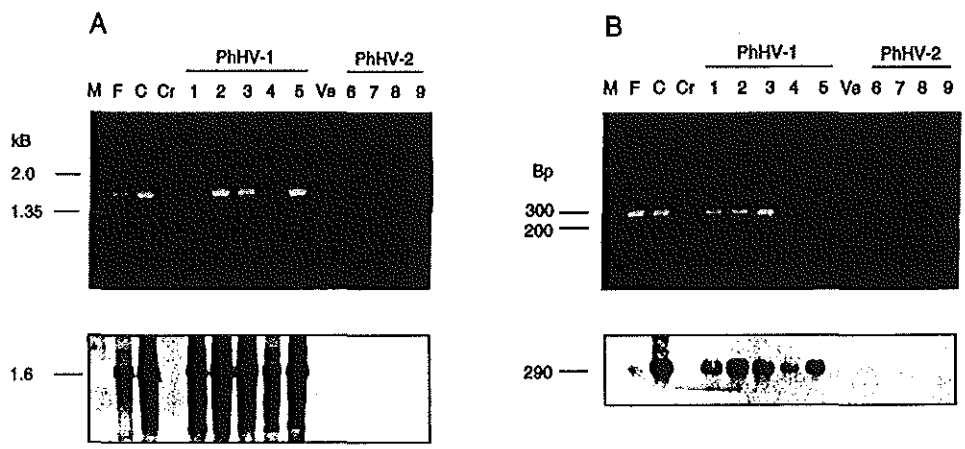
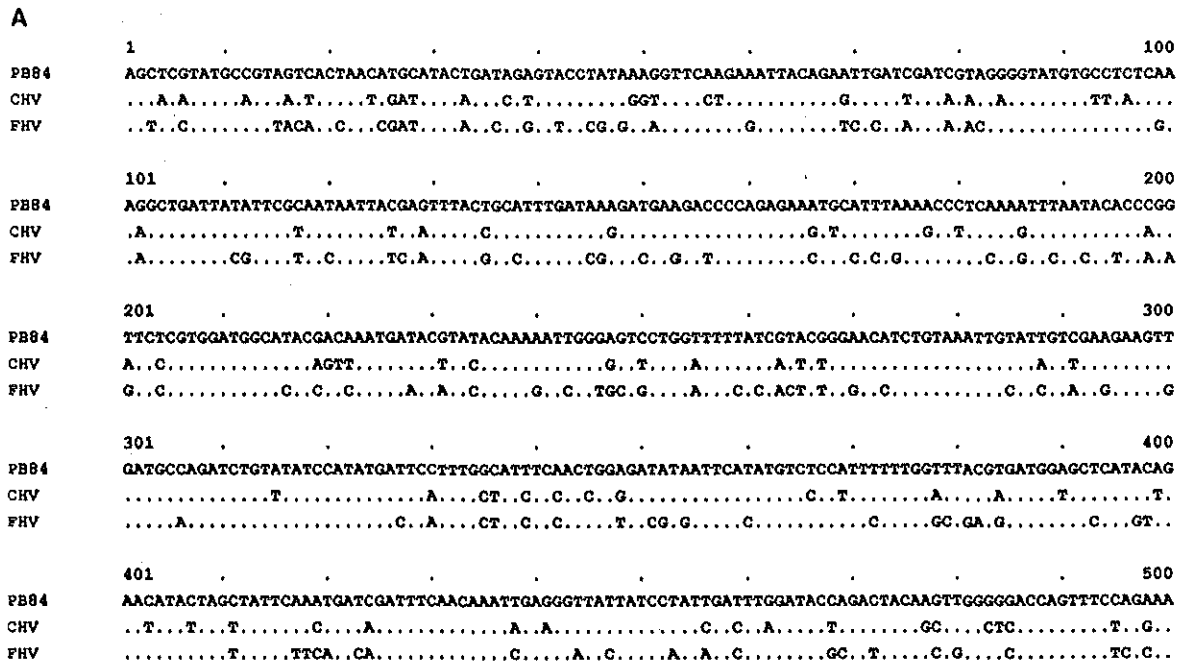


Figure 2. Amplification by PCR and detection by non-radioactive Southern blotting of 1.6 kB gB (A) and 290 bp gD (B) gene equivalent fragments from various pinniped herpesviruses and herpesviruses of terrestrial carnivores. Template DNA was extracted from infected cell cultures and probes were prepared from cloned gB (A) and gD (B) fragments, respectively, of the PhHV-1 isolate PB84. Washing steps were carried out under moderately (A) or low (B) stringent conditions. M - marker, F - FHV 6887/Han90, C - CHV 5105/Han89, Cr - uninfected CrFK cells, 1 - PB84, 2 - 2557/Han88, 3 - Ja/PB94, 4 - 550/Hel93, 5 - A92/10-4, Ve - uninfected Vero cells, 6 - 7848/Han90, 7 - Zalo/Cal86, 8 - A92-10/5, 9 - A93-2/11.

Figure 3 (opposite page). Sequence alignments (excluding primer sequences) of 500 nucleotides from the 5'-end of a 1.6 kB gB gene fragment of PhHV-1 PB84 (A) and of a 256 bp fragment of the gD equivalent gene amplified by PCR from several PhHV-1 isolates (B). Alignments of sequences were created with 'PileUp' of the GCG software package (release 8.0) setting gap creation weights to 3.0 and gap extension weights to 0.1. The sequences of felid herpesvirus (FHV-gB: #S49775, FHV-gD: #D30767) were extracted from the GenBank database; the sequences of canid herpesvirus gB and gD were taken from Limbach *et al.*, 1994. Nucleotides identical to the sequence of PhHV-1 PB84 are shown as dots. Alignment gaps are indicated by dashes (-).


A
1 100
PB84 AGCTCGTATGCCGTAGTCACTAACATGCATACTGATAGAGTACCTATAAAGGTTCAAGAAATTACAGAATTGATCGATCGTAGGGGTATGTGCCTCTCAA
CHV ...A.A.....A...A.T.....T.GAT....A...C.T.........GGT....CT...........G.....T...A.A..A.........TT.A....
FHV ..T..C.........TACA..C...CGAT....A..C..G..T..CG.G..A.........G.........TC.C..A...A.AC...............G.
101 200
PB84 AGGCTGATTATATTCGCAATAATTACGAGTTTACTGCATTTGATAAAGATGAAGACCCCAGAGAAATGCATTTAAAACCCTCAAAATTTAATACACCCGG
CHV .A..............T.........T..A.....C...........G.................G.T.........G..T.....G...........A..
FHV .A.........CG....T..C.....TC.A.....G..C......CG...C..G..T.........C...C.C.G.........C..G..C..C..T..A.A
201 300
PB84 TTCTCGTGGATGGCATACGACAAATGATACGTATACAAAAATTGGGAGTCCTGGTTTTTATCGTACGGGAACATCTGTAAATTGTATTGTCGAAGAAGTT
CHV A..C..............AGTT.........T..C.............G..T....A.......A.T.T.....................A..T.........
FHV G..C...........C..C..C.....A..A..C.....G..C..TGC.G....A...C.C.ACT.T..G..C...........C..C..A..G.....G
301 400
PB84 GATGCCAGATCTGTATATCCATATGATTCCTTTGGCATTTCAACTGGAGATATAATTCATATGTCTCCATTTTTTGGTTTACGTGATGGAGCTCATACAG
CHV ..............T..............A....CT..C..C..C..G.................C..T.........A.....A.....T.........T.
FHV .....A...................C..A....CT..C..C.....T..CG.G.....C...........C.....GC.GA.G.........C...GT..
401 500
PB84 AACATACTAGCTATTCAAATGATCGATTTCAACAAATTGAGGGTTATTATCCTATTGATTTGGATACCAGACTACAAGTTGGGGGACCAGTTTCCAGAAA
CHV ..T...T...T.......C....A.............A..A..............C..C..A.....T.........GC....CTC.........T..G..
FHV ..........T.....TTCA..CA.............C.....A..C.....A..A..C.........GC..T.....C.G....C.........TC.C..
B
1 100
PB84 ATCTACAGATCCATGTGGTATGGTTGCTTTAATTTCTGAGCCAAATATAGAATCTACAATTAAAACTATTCAATTTGTAAATAAAAAAAAATATTATAAC
Ja/PB94 ....
550/Hel93 ....T
A92-10/4 ....
CHV T.....T..C.......A.............G......A.T.........................G...........GC.A..G.......T...C..T
FHV ...A.TG...T.......G.....G..A..G..A..A.....GG.......CG....T...CG...C..A...---C..TC.C.........ACA......
101 200
PB84 GCTTCGCTTAGTTGGTTTAAAGTTGGAGATGATTGTACATATCCAATATACTTAATTAAATATTTTAATTGCGATCCTCAAAAAGAGTTTGGTATATGCT
Ja/PB94 ....G...
550/Hel93 ....
A92-10/4 ....
CHV ..A..T...T......C.........G....T..........G...A.....C.....T.
FHV ..GA.TA.A...........G..AACCC.G.G....GA...C..T..G.TTC.T..GG.TATGAGACT...T......A..CGG..A.....A.....TG
201 256
PB84 TAAAAAGAACACCCGATTATTGGAAACCATCATTGATTGGTTATTCTTTTTTAACA
Ja/PB94 ....
550/Hel93 ....
A92-10/4 ....
CHV .........T.T..A....T............G..AG.......CA.A........T
FHV CTTT.C.GT.G..TTCA......TTGGA.C.T..A.CAAAG...ATG..CC.....

Amplification, cloning and sequencing of a 290 bp gD gene fragment of PhHV-1

Amplification of a 290 bp fragment from the gD gene equivalent of PhHV-1, CHV and FHV, but not from PhHV-2, was achieved using primers gD_1 – gD_2, employing conditions identical to those used for the gB fragment. Southern blotting using the gD fragment of PhHV-1 PB84 as a probe revealed that under low stringent conditions significant hybridization was achieved only with PhHV-1 isolates and CHV (Fig. 2B).

Amplificates of four PhHV-1 isolates (PB84, 550/Hel93, Ja/PB94 and A92-10/4) were cloned and sequenced to detect possible strain differences. The PhHV-1 isolates, regardless of their geographic or host origin, were highly conserved with only single nucleotide variations in the analysed region (Ja/PB94, 550/Hel93). The sequences of PB84 and A92-10/4 were identical. An alignment of these sequences with corresponding regions of FHV and CHV gD genes is presented in Fig. 3B and shows that in this gene fragment, PhHV-1 shares considerable nucleotide identity with CHV (86%) and to a lesser degree with FHV (61%).

Cloning and sequencing of EcoRI fragments of the PhHV-2 genome

The nucleotide and deduced amino acid sequences of two *Eco*RI fragments of 788 (A) and 315 bp (B), respectively, are presented in Fig. 4. Full-length open reading frames (ORFs) extending beyond the cloned fragments were identified using the option 'Frames' included in the GCG software package. A 'FastA' (GCG) database search employing the deduced protein products revealed significant homologies to proteins of γ-herpesviruses (Table 3). The ORF identified in fragment A was shown to be equivalent to the product of the *UL52'* gene family of herpesviruses which encodes a part of the helicase-primase complex (Crute *et al.*, 1989). This gene is conserved between all herpesvirus subfamilies. Its representative in γ-herpesviruses is embedded in a gene cluster (referred to as block V) which is conserved between the herpesvirus subfamilies with respect to the collinear arrangement of the ORFs (Albrecht *et al.*, 1992).

The ORF identified in clone A mapped to a different region of the γ-herpesvirus genome, namely the BTRF1 gene of Epstein-Barr virus and its equivalents in EHV-2 and herpesvirus saimiri type-2 (Table 3). This gene is interspersed between blocks II and III and appears to have no counterpart in α- or β-herpesviruses (Albrecht *et al.*, 1992).

When comparing the similarity and identity values of the deduced amino acid sequences, highest scores were generally found between PhHV-2 and EHV-2 (Table 3).

Figure 4 (opposite page). Nucleotide and deduced amino acid sequences of a 788 (A) and a 315 bp (B) *Eco*RI fragment of the PhHV-2 (isolate 7848) genome. Open reading frames were identified using the option 'Frames' included in the GCG software package (release 8.0) (Devereux *et al.*, 1984).

A

```
   1  N  S  E  S  P  N  L  M  L  Q  E  E  L  Q  G  L  L  D  F  A    20
   1  GAATTCGGAATCTCCAAACCTGATGCTACAAGAGGAGCTCCAGGGCCTCTTGGATTTTGC    60

  21  A  T  A  P  D  D  G  R  N  C  T  K  G  D  I  F  P  T  V  H    40
  61  AGCCACTGCGCCAGATGATGGCAGAAACTGCACTAAAGGTGATATCTTTCCTACAGTGCA   120

  41  I  N  P  V  F  R  C  Q  F  L  N  K  N  Y  F  V  I  V  N  A    60
 121  CATCAACCCCGTGTTCAGATGCCAGTTCCTAAACAAAAACTACTTTGTGATTGTTAATGC   180

  61  D  I  L  T  Q  V  W  K  S  T  V  L  L  P  Q  T  P  N  W  A    80
 181  AGACATATTAACTCAGGTATGGAAAAGCACTGTGCTCCTACCGCAAACTCCCAACTGGGC   240

  81  T  T  L  T  D  M  Q  I  T  E  R  I  F  Y  K  E  T  F  F  S   100
 241  TACAACTCTAACAGATATGCAGATAACTGAGCGCATTTTTTACAAAGAGACATTTTTCTC   300

 101  L  N  N  I  K  D  Q  L  Q  I  S  R  H  E  Y  F  N  V  R  V   120
 301  CTTAAACAACATCAAGGATCAGCTTCAGATATCAAGGCACGAGTACTTTAATGTGAGGGT   360

 121  P  V  F  N  L  V  L  D  F  D  L  P  L  G  V  K  G  L  T  L   140
 361  GCCAGTTTTCAACCTGGTGTTAGACTTTGACCTGCCCCTTGGTGTGAAAGGTCTCACTCT   420

 141  H  Q  I  Y  D  V  C  L  A  L  R  E  D  V  I  Q  I  L  Q  L   160
 421  CCATCAAATTTACGATGTGTGCTTGGCTCTCCGAGAGGACGTCATACAGATTTTGCAGCT   480

 161  L  G  D  V  D  P  Q  T  H  Q  V  Y  F  F  K  S  S  C  P  P   180
 481  ATTGGGTGATGTTGATCCCCAGACACACCAGGTGTATTTTTTTAAGTCATCGTGCCCCCC   540

 181  L  E  W  D  L  D  E  K  M  F  C  N  C  S  E  K  L  G  L  R   200
 541  TCTAGAGTGGGACTTGGATGAAAAAATGTTCTGCAACTGCTCTGAAAAGCTAGGACTCAG   600

 201  V  V  T  N  L  P  R  G  T  A  I  V  G  S  E  P  L  I  T  L   220
 601  GGTTGTGACAAACCTTCCGAGAGGTACTGCTATTGTTGGATCAGAGCCTCTGATCACTCT   660

 221  V  K  I  L  N  R  M  V  K  M  N  P  L  F  L  H  L  C  P  T   240
 661  AGTAAAGATTTTAAACAGGATGGTCAAAATGAATCCTCTATTTTTACATCTCTGCCCCAC   720

 241  L  L  D  S  E  G  L  L  I  L  E  F  T  I  R  E  R  K  C  V   260
 721  ACTCCTAGATAGCGAAGGCCTTTTGATTCTGGAATTTACCATAAGGGAAAGGAAGTGTGT   780

 261  R  I     262
 781  CAGAATTC  788
```

B

```
   1  N  S  T  L  T  P  I  Q  T  L  F  I  K  H  V  L  L  K  K  M    20
   1  GAATTCTACACTCACCCCAATACAAACACTCTTTATAAAACATGTGTTGTTAAAGAAAAT    60

  21  G  L  E  N  C  I  T  D  F  Q  N  L  Y  N  P  H  L  S  T  I    40
  61  GGGGCTTGAGAATTGCATTACAGATTTTCAAAACCTCTACAACCCTCACCTCTCCACAAT   120

  41  S  E  S  Q  L  L  E  F  G  K  L  V  A  E  A  K  G  R  V  E    60
 121  ATCTGAGAGCCAGTTGCTGGAGTTTGGAAAGCTGGTAGCAGAAGCCAAGGGCCGTGTGGA   180

  61  D  I  M  F  A  L  N  S  I  S  Q  A  T  F  S  K  P  V  L  P    80
 181  AGATATCATGTTTGCTCTAAATTCAATATCACAAGCCACATTTTCAAAGCCTGTGTTGCC   240

  81  G  T  D  V  Q  C  V  M  M  M  E  K  Y  F  W  M  F  P  P  V   100
 241  AGGAACAGACGTGCAATGTGTTATGATGATGGAGAAGTACTTTTGGATGTTCCCACCCGT   300

 101  D  P  M  N     105
 301  TGATCCCATGAATTC  315
```

Table 3: Relationships of amino acid sequences predicted from two *Eco*RI fragments of the PhHV-2 isolate 7848.

PhHV-2 fragment	Virus species	Gene equivalent[a]	Comparison of ORFs [b]		
			%Similarity	%Identity	Gaps
*Eco*RI 788 bp (262 aa)	Equine herpesvirus-2	ORF 56	63.6	47.9	4
	Herpesvirus saimiri-2	EDRF4	67.7	46.5	3
	Epstein-Barr virus	BSLF1	60.0	36.5	5
	Human cytomegalovirus	UI70	54.0	33.2	9
	Equine herpesvirus-1	ORF 7	55.0	29.2	13
	Varicella zoster virus	ORF 6	49.8	28.6	11
*Eco*RI 315 bp (105 aa)	Equine herpesvirus-2	ORF 23	69.2	44.2	1
	Epstein-Barr virus	BTRF1	62.5	42.3	1
	Herpesvirus saimiri-2	ORF 23	54.8	35.6	1

[a] Sequences for comparisons were extracted from the SwissProt or the GenBank databases: EHV-2 #U20824; HVS-2 #Q01006, #P14346; EBV #P30119, #P03193; HCMV #P17149; EHV-1 #P28962; VZV #P09270.

[b] Deduced amino acid sequences were compared by using the 'Gap' program of the GCG software package, setting gap weight and gap extension values to 3.0 and 0.1, respectively. The calculation of similarity values is based on the Dayhoff matrices (Dayhoff *et al.*, 1983).

Discussion

Herpesvirus isolates obtained during the last decade from different pinniped species in widely separated populations were analysed. Two types of pinniped herpesviruses could be readily distinguished by means of their antigenic and genetic properties. As all but one of the isolates were retrieved from species of the family *Phocidae* their designation as *phocid* herpesvirus (PhHV) type-1 and type-2 seems to be justified. Both types were isolated from seals ranging in European waters as well as in coastal United States waters. While no antigenic differences between American and European PhHV-1 isolates were observed, the European PhHV-2 isolate 7848/Han88 differed in at least one epitope from the American PhHV-2 viruses. The PhHV-2 isolate Zalo/Cal86, retrieved from a captive Californian sea lion, a member of the family *Otariidae*, was not distinguishable from other American PhHV-2 isolates of harbour seal origin by means of MAb typing. Interspecies transmission from clinically healthy harbour seals, which were housed in the immediate vicinity of the sea lion could not be excluded. However, PhHV-2 has so far only been isolated from cultivated leucocytes (Lebich *et al.*, 1994; this study) or explant lung tissue cultures (Kennedy-Stoskopf *et al.*, 1986), which

together with its replication kinetics in CrFK cells indicates that its infectivity is highly cell-associated. This does not favour interspecies transmission as a likely cause of infection of the sea lion. The significance of the fact that PhHV-1 has mainly been isolated from European seals while all but one of the American isolates have been identified as PhHV-2 cannot be readily explained at present. Type-specific serosurveys could be performed to gain more insight in the prevalences of the different pinniped herpesvirus types.

The use of partially degenerated primers selected from conserved regions of the gB and gD homologous genes of CHV, FHV, and EHV-1 enabled the amplification of gB and gD specific sequences from PhHV-1 by PCR. No fragments were amplified from PhHV-2. This emphasizes once more the more distant relationship of PhHV-2 to these viruses. We have taken advantage of the PhHV-1 gD PCR amplification for diagnostic purposes, by showing that it was more sensitive in diagnosing acute PhHV-1 infections than virus isolation procedures, carried out with clinical samples from harbour seals during an acute outbreak of respiratory disease. This newly developed PCR analysis may also be useful for the diagnosis of CHV and FHV infections (T. Harder and others, unpublished). Sequencing of a 290 bp fragment of the gD gene of several PhHV-1 isolates showed that, in spite of their different geographic and host origins, these viruses were identical except for a single nucleotide in that fragment. By comparison with gD genes of a number of herpesviruses, PhHV-1 was shown to share a high degree of homology with members of the genus *Varicellovirus* and CHV in particular. Similar results were found with gB specific sequences of the PhHV-1 isolate PB84. These results confirm earlier studies on the serological characterization of PhHV-1 as a putative member of the genus *Varicellovirus* (Osterhaus *et al.*, 1985; Frey *et al.*, 1989; Harder *et al.*, 1991a; Stenvers *et al.*, 1992b; Lebich *et al.*, 1994).

The taxonomic status of PhHV-2 was elucidated by sequence analysis of two *Eco*RI fragments. A clear clustering with γ-herpesviruses was found on the basis of a database search using the deduced amino acid sequences of single ORFs identified in each fragment. The sequences obtained proved to be unique when compared to other γ-herpesvirus species where equivalent sequence data were available (Epstein-Barr virus, herpesvirus saimiri type-2 and EHV-2). PhHV-2, therefore, could represent a hitherto undescribed γ-herpesvirus. On the basis of the limited sequence data, the closest relative of PhHV-2 amongst the γ-herpesviruses could not be unambiguously identified, although the highest similarity/identity scores were mainly encountered with EHV-2. Additional sequence data from different genome parts will be required for a definite taxonomic grouping. A clinical correlate of PhHV-2 infection in seals has not been identified so far.

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Section 2.3

Age-related disease in recurrent outbreaks of phocid herpesvirus type-1 infections in a seal rehabilitation center: Evaluation of diagnostic methods

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Abstract — The prevalence and clinical signs of phocid herpesvirus type-1 (PhHV-1) infections amongst harbour seals (*Phoca vitulina*) in a seal rehabilitation center in the Netherlands were monitored between June and September 1993 and 1994 when 34 and 36 seals, respectively, were rehabilitated. In both years PhHV-1 related disease outbreaks occurred in the pupping season. PhHV-1 infections were diagnosed by demonstration of a more than four-fold increase in virus neutralizating serum antibodies in paired serum samples, by the isolation of the virus from swab samples in primary seal kidney cells, and by the detection of PhHV-1 DNA with a polymerase chain reaction (PCR) assay in swab samples. This assay targets a 290 bp fragment of the glycoprotein D (gD) gene equivalent of PhHV-1. The PCR assay when combined with Southern blotting (PCR-SB) was approximately thousand times more sensitive than virus isolation when tested with serially diluted samples from PhHV-1 infected cell cultures. In contrast with virus isolation, the PCR-SB scored as positive all the animals with serological evidence of PhHV-1 infection. The majority of seals present in the center during the outbreaks contracted the infection and developed benign upper respiratory disease. However, the severity of PhHV-1 related disease was inversely correlated with age and fatal generalized infections occurred only in neonates.

Introduction

Two types of pinniped herpesviruses have been distinguished. They were recovered from members of the *Phocidae* family and are referred to as phocid herpesviruses type-1 and type-2 (PhHV-1, -2) (Osterhaus *et al.*, 1985; Frey *et al.*, 1989; Lebich *et al.*, 1994). PhHV-1 was shown to be antigenically closely related to felid and canid herpesviruses (Osterhaus *et al.*, 1985; Lebich *et al.*, 1994), whereas PhHV-2 appeared to be more distantly related to both PhHV-1 and herpesviruses of terrestrial carnivores: Recently, nucleotide sequence data have been obtained for PhHV-1 and PhHV-2 which suggest a taxonomic clustering of these viruses within the *Varicellovirus* genus of the *α-Herpesvirinae* subfamily and within the *γ-Herpesvirinae*, respectively (Harder *et al.*, 1996a).

Infections with PhHV-1 in harbour seals (*Phoca vitulina*) have been associated predominantly with benign upper respiratory disease, but an enzootic outbreak with high mortality among newborn seals has been described in the population present at a seal rehabilitation and research center (Osterhaus *et al.*, 1985; Borst *et al.*, 1986). During the 1988 seal mass mortality in north west Europe, caused by phocid distemper virus (PDV), generalized PhHV-1 infections were frequently detected in fatally diseased, PDV-infected adult seals (Osterhaus & Vedder, 1988; Frey *et al.*, 1989; Zhang *et al.*, 1989). Upon experimental inoculation with a tissue culture-propagated PhHV-1 isolate subadult harbour seals seroconverted, but apart from a moderate temperature rise no overt clinical symptoms developed (Horvat *et al.*, 1989). No association of PhHV-2 infection with disease in pinniped populations has so far been reported.

The paper describes investigations of the prevalence and clinical signs of PhHV-1 infection among harbour seals of different ages at the seal rehabilitation and research center of Pieterburen in 1993 and 1994, and compares the result of different diagnostic methods.

Materials and methods

Seals – The center aims to rehabilitate weakened, orphaned or diseased seals which are found along the Dutch coast. The majority of the animals rehabilitated at the center are newborn harbour seals or grey seals *(Halichoerus grypus)*, which are admitted during pupping seasons in June/July and December, respectively. During the present study, an additional group of 22 adult harbour seals, all seropositive for PhHV-1, was kept at the center, for immunotoxicological investigations (De Swart *et al.*, 1994). However, animals of this group were physically separated from the seals housed for rehabilitation. The survey was restricted to 52 harbour seals and one ringed seal *(Pusa hispida)*. Thirty-three harbour seals were present at the center between June and October 1993 and 19 during the same period of 1994. From each animal a complete set of samples (swabs [eyes, nose and throat], a heparin blood sample and paired serum samples) was available. The total numbers of seals present at the center at these times were 34 and 36, respectively.

Detection of PhHV infection by virus isolation and serological assays – Virus isolation procedures were applied to swab samples using primary harbour seal kidney cells (SeKC) as described by Osterhaus *et al.* (1985). Isolates were subsequently antigenically characterized using a recently established panel of murine monoclonal antibodies (MAbs) in an immune-peroxidase monolayer assay (IPMA) (Lebich *et al.*, 1994).

Virus neutralizing (VN) antibody titers against the PhHV-1 isolate PB84 were measured in paired serum samples as described (Osterhaus *et al.*, 1985). A more than four-fold rise of PhHV VN titer was scored as evidence for active PhHV-1 infection.

All swab samples were also checked for evidence of morbillivirus infections as previously described by Visser *et al.* (1989).

Origin of clinical specimens for the evaluation of a PCR assay – Nose, throat or eye swabs were collected from six harbour seals and one ringed seal, which were hospitalized in 1993 or 1994, during outbreaks of upper respiratory disease. Retrospectively, these seals were shown to have experienced active PhHV-1 infection on basis of seroconversion and/or virus isolation. In addition, throat swabs from ten harbour seals which proved to be negative in virus isolation assays and did not seroconvert to PhHV-1 during the outbreaks were included. As controls, nasal and throat swab samples of six clinically healthy grey seal pups were sampled in 1995 when no signs of PhHV-1 related disease were observed in the seal population at the center. These grey seals did not seroconvert to PhHV-1 during their stay at the center.

The swabs were kept refrigerated in 4 ml of DMEM supplemented with penicillin (300 i.u ml^{-1}) and streptomycin (300 μg ml^{-1}). An aliquot (1.0 ml) was inoculated into SeKC and Vero cell cultures for virus isolation within four days after sampling. The remaining fluid was frozen at -80°C for up to fifteen months until used for DNA isolation as described below.

Preparation of DNA for PCR assay – DNA was recovered from diagnostic swab samples of seals by adapting a silica-based technique as described by Boom *et al.* (1990). Briefly, 100 μl of a sample were mixed with 1 ml of lysis buffer (120 g guanidine isothiocyanate in 100 ml 0.1 M Tris HCl [pH 6.4], to which 22 ml 0.2 M EDTA [pH 8], 2.6 g Triton X 100 and 40 μl acidified Celite silica (Janssen Chimica) were added. After incubation for 10 minutes at room temperature the silica was pelleted and washed twice in lysis buffer lacking EDTA and Triton. Additional washing steps in 70% ethanol, and acetone, were carried out, after which the silica pellet was vacuum-dried. DNA was

released by adding of 100μl of TE-buffer (10mM Tris HCl pH 8.0, 1mM EDTA) and incubating at 56°C for ten minutes. For the PCR, aliquots of 20 μl were used.

As a control, PhHV-1 DNA (reference isolate PB84) was prepared from infected Crandell Rees feline kidney (CrFK) cell cultures as described by Harder *et al.* (1996a).

Primer selection and polymerase chain amplification – Primers were selected from conserved regions of the gD equivalent genes of felid and canid herpesvirus (Limbach *et al.*, 1994; Maeda *et al.*, 1994), which were found to be the closest relatives of PhHV-1 at the antigenic level (Lebich *et al.*, 1994). The DNA PCR, using primers gD_1 (5'- gAA gTT Cgg TAT gT[A/T] AC - 3') and gD_2 (3'- gA[C/T] gAT gAA TTg ggA TT - 5') was essentially as described (Harder *et al.*, 1996a), and produced specific amplificates of 290 bp from PhHV-1 but not from PhHV-2 templates.

The products were separated on agarose gels and visualized after staining with ethidium bromide. Non-radioactive Southern blotting was used to confirm the specificity of the amplificates (Harder *et al.*, 1996a). A cloned gD fragment of the PhHV-1 reference isolate was used as a probe in chemoluminiscent assays (ECL, Amersham).

Measures to minimize the risk of carry-over contaminations consisted of physical separation of pre- and post-PCR procedures, the preparation of templates in a laminar flow cabinet, and the use of master mixes and aerosol-resistant tips. Negative controls (bidistilled water or tissue culture medium) were routinely run in parallel.

Clinical scoring of PhHV-1 related disease – The serological results obtained from an examination of paired serum samples were used as a 'golden standard' for diagnosis of active PhHV-1 infection. Seals with a serologically confirmed PhHV-1 infection during the 1993 and 1994 outbreaks were grouped arbitrarily according to the severity of the disease. Group 0 seals showed no clinical signs. Seals of group 1 showed mild clinical signs consisting of anorexia, mucous nasal and/or ocular discharge and rectal temperatures of up to 38.5°C. These seals recovered completely within one week at most. Group 2 animals suffered more severe signs of disease, including mucupurulent nasal and ocular discharge, epistaxis, lower respiratory disease (dyspnoe, coughing), and in some cases gastrointestinal symptoms such as vomiting and diarrhea. Their rectal temperatures frequently exceeded 38.5°C. They recovered completely with palliative antibiotic treatment within two weeks. The seals which succumbed to the infection were classified in group 3.

Results

Virus isolation and serology

Outbreaks of an apparently contagious disease, mainly characterized by upper respiratory symptoms, were noticed among the harbour seal pups during the summer months of both years. In 1993 an active PhHV-1 infection was confirmed by seroconversion in 24 of 34 seals of all age groups (Tab. 1).

Nineteen of these animals developed overt clinical symptoms. Ten seals were apparently not infected during their stay at the center and did not show clinical symptoms suggestive of the infection. Most of the PhHV-1 isolates were recovered from swab samples. In some animals swabs from eyes, nose and throat proved virus isolation positive, but in others only

Table 1. Detection of active PhHV-1 infection in captive harbour seal populations.

Season	PhHV-1 isolation	Seroconversion	Disease
June-August '93	10[a]/34[b]	24/34	19/24[c]
August-September '94	14/19	19/19	18/19

[a] Number of positive animals; [b] Number of animals examined; [c] Number of animals with serologically confirmed active PhHV-1 infection.

one swab sample was positive with no clear preference for the location. Leukocyte-associated viremia could not be detected in any of the animals studied. However, in three newborn seals which succumbed to the disease in 1993, generalized PhHV-1 infection was demonstrated by virus isolation from different tissue specimens including lung, kidney, liver or CNS.

Within the group of 22 PhHV-1 seropositive adult seals kept separate from other seals, no clinical signs indicative of PhHV-1 infection were observed during the outbreaks. However, in seven adult seals more than four-fold increases in PhHV-1 VN titer coincided with PhHV-1 related disease outbreaks in the groups of seal pups.

The patterns of reaction of the panel of 32 MAbs with the PhHV-1 isolates obtained in 1993 and 1994 were identical to those obtained with the strains isolated in 1984 (Osterhaus *et al.*, 1985) and during the mass mortality of seals in 1988 (Lebich *et al.*, 1994; data not shown).

No morbilliviruses were isolated from the materials used for PhHV-1 isolation.

Diagnosis of PhHV-1 infection by PCR

To determine the sensitivity of the PhHV-1 PCR assay, serial ten-fold dilutions of supernatant from a CrFK cell culture infected with PhHV-1 PB84 were examined. As shown in Fig. 1, a positive signal was still obtained in the $10^{-5.0}$ diluted sample. Titration of the same serial ten-fold dilutions in CrFK cells revealed a titer of $10^{1.9}$ $TCID_{50}$ per 20 μl of the undiluted stock. Thus, the detection limit of the PCR assay with Southern blotting (PCR-SB) was $10^{-3.0}$ $TCID_{50}$.

The suitability of this PCR assay for the detection of PhHV-1 DNA in clinical specimens was investigated with swab samples from the eye, nose or throat of six harbour seals and one ringed seal which had all seroconverted to PhHV-1 (Fig. 2, panel A). The gD fragment was detected in all the samples from which PhHV-1 had been isolated. Furthermore, six samples which were virus isolation negative were positive in PCR-SB. All seals, except the adult harbour seal PB93-Cath. and the subadult ringed seal of this group showed signs of respiratory disease at the time of sampling, indicating that they were asymptomatic virus shedders.

PhHV-1 DNA was not detected by PCR-SB in swabs taken from six clinically healthy grey seal pups sampled as controls when no evidence for a PhHV-1 infection was found (Fig. 2, panel B). Swab samples from ten further clinically healthy harbour seals of the 1993 season which remained negative in virus isolation assays and did not seroconvert to PhHV-1 also scored negative in the PCR-SB (not shown).

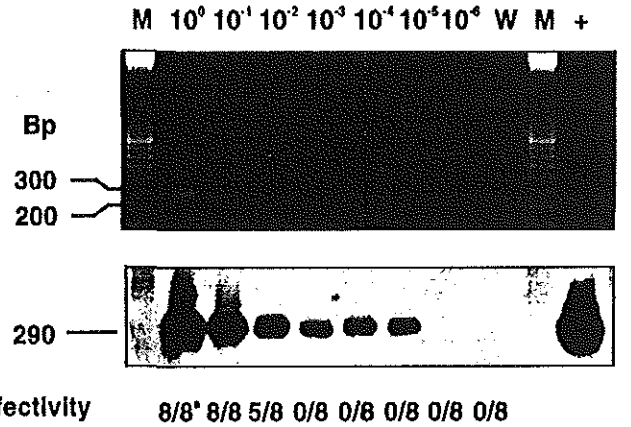

Figure 1. Detection limits of a PCR targeting a 290 bp fragment of the gD gene of PhHV-1 combined with Southern blotting. Template DNA was purified from 20 μl of serial tenfold dilutions of a supernatant from a CrFK cell culture infected by PhHV-1 PB84. A parallel titration of viral infectivity (bottom line) revealed a titer of $10^{1.9}$ $TCID_{50}$ per 20 μl of the undiluted stock. W: cell culture medium control, +: corresponding gD fragment of PhHV-1 isolate PB84 amplified in a separate PCR reaction. Non-radioactive Southern blotting was carried out with a probe prepared from the cloned gD fragment of PhHV-1 PB84.

[a] Number of virus-positive/total wells in a microtitration assay based on the development of cytopathic alterations.

Correlation of PhHV-1 disease severity with age

In Fig. 3 the seals with serologically confirmed active PhHV-1 infection are grouped according to their age and the severity of their clinical symptoms. Pups up to one month old were distinguished from juveniles aged one to twelve months and subadults or adults more than twelve months old. The severity of PhHV-1 related disease appeared to be inversely correlated with age. The fatal cases were restricted to three newborn animals.

Figure 2. PCR amplification and non-radioactive Southern blot detection of a 290 bp fragment of the gD gene equivalent of PhHV-1 from diagnostic swab samples obtained from from harbour seals (*Phoca vitulina*) and a ringed seal (*Pusa hispida*) during outbreaks of respiratory symptoms in a seal sanctuary in 1993/1994 (panel A). Clinically healthy grey seal pups (*Halichoerus grypus*) were sampled as controls in 1995 when no evidence of PhHV-1 related disease was noticed in the sanctuary's seal population (panel B). DNA was prepared from 100 μl of swab fluid. An equivalent of 20 μl was used for PCR. The results of virus isolation attempts on SeKC cultures using 1 ml of fresh swab fluids is indicated in the bottom line. Before use in PCR, samples of panel A had been stored at -80°C for up to 15 months while other samples were processed within four days after sampling. (Continued overleaf...)

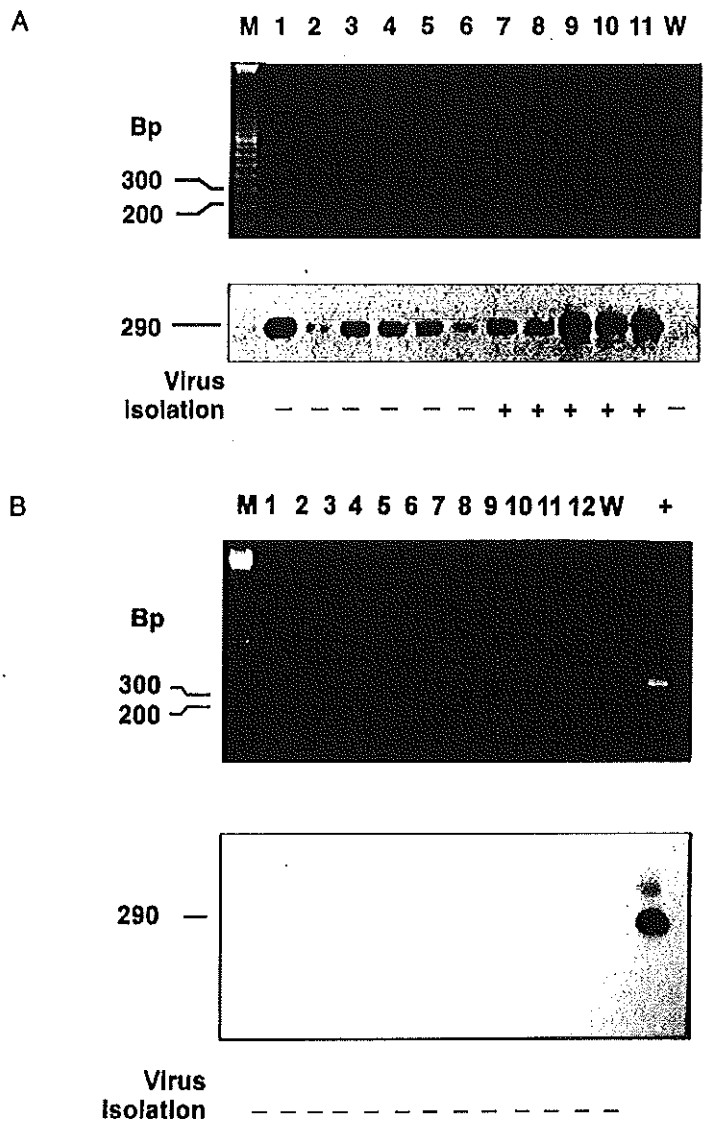

Figure 2 (continued from previous page):
Panel A: 1,2 - *Pusa hispida* PB94-15 (throat, eye); 3 - 7: samples from *Phoca vitulina*: 3 - PB94-22 (nose); 4 - PB94-23 (throat); 5 - PB94-24 (nose); 6 - PB94-40 (nose); 7,8 PB93-Jac. (nose, eye); 9,10,11 PB93-Cath. (nose, throat, eye); W - Swab medium control.
Panel B: All swab samples (throat, nose) from *Halichoerus grypus*: 1,2 - PB95-27; 3,4 - PB95-12; 5,6 - PB95-04; 7,8 - PB95-02; 9,10 - PB95-30; 11,12 - PB95-07; W - Swab medium control; + - DNA sample extracted from CrFK cells infected by PhHV-1 isolate PB84.

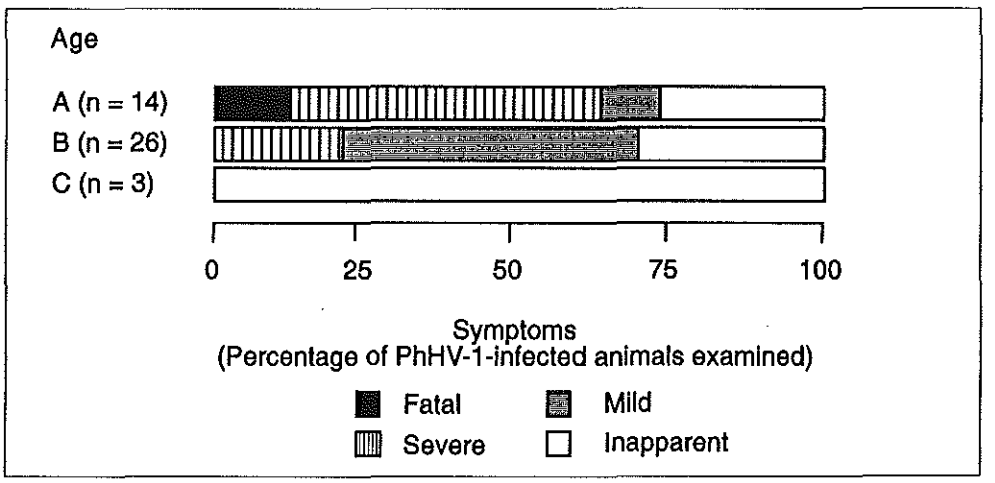

Figure 3. Disease symptoms associated with PhHV-1 infection in harbour seals of different age groups. Clustering according to the disease symptoms is described in Materials and methods. Three age groups were distinguished: A - neonates: up to one month, B - juveniles: one to twelve months and C - subadults and adults older than twelve months.

Discussion

Three different methods for the laboratory diagnosis of PhHV-1 infections in pinnipeds have been compared in animals in which the clinical signs were largely dependent on the age. During the two pupping seasons studied PhHV-1 was by far the most frequently detected viral agent in this population, which is routinely vaccinated against PDV (Visser *et al.*, 1989). The inverse correlation between PhHV-1 associated disease and age is reminiscent of α-herpesvirus infections of terrestrial carnivores, for example canid herpesvirus (Appel 1987). The results are also in line with earlier studies in which the experimental PhHV-1 inoculation of susceptible juvenile harbour seals resulted in no apparent infection (Horvat *et al.*, 1989). The asymptomatic PhHV-1 infections observed in the adult seals were most probably due to the reactivation of latent virus. During the 1993 outbreak three newborn pups (12.5% of actively PhHV-1 infected seals) died in the course of the infection. This mortality rate is low when compared to the first recognized PhHV-1 enzootic in 1984, when up to 50% of the diseased neonates died (Osterhaus *et al.*, 1985). Although it cannot be excluded that PhHV-1 strains with different pathogenic potentials may have been co-circulating and that the recent outbreaks may have been caused by milder strains, no significant differences in the antigenic make-up between the isolates obtained in 1984 and in 1993/94 were detected. Howver, since 1984 knowledge of and experience with quarantine measures, veterinary care and nursing of seal pups have greatly improved, and the changes may have contributed to the reduction in losses. They may also explain why in 1993 10 out of 34 seals did not become infected during their stay at the center.

The origin of the PhHV-1 strains responsible for the outbreaks could not be identified. As in herpesvirus infections of terrestrial carnivores (Appel 1987), pups may be infected directly after birth and thus may have carried the virus when they were admitted to the center. Although experimental evidence for latency of PhHV-1 in seals is lacking, the epidemiological importance of seals which are seropositive to PhHV-1 after loss of maternal antibodies, should not be underestimated, because these animals may be latent virus carriers and shed the virus after reactivation.

The accurate etiological diagnosis of herpesvirus infections in marine mammals by virus isolation procedures is often hampered by the unsuitability of many diagnostic samples as a result of advanced decay and bacterial contamination. The PhHV-1 gD-specific PCR-SB was approximately thousand-fold more sensitive than virus isolation. This factor may even be higher when clinical specimens are analysed after storage at suboptimal conditions, which would be expected to affect herpesviral infectivity more than the integrity of template DNA. Indeed, the PCR-SB was more sensitive than virus isolation procedures in SeKC cultures for detection of PhHV-1 infection in archived clinical specimens from seals. The limited accessibility of permissive cell cultures is another reason why PCR-SB may be favoured for the rapid diagnosis of active PhHV-1 infection in individual seals. The reported inability to diagnose PhHV-2 infections in this way (Harder *et al.*, 1996a) is of less clinical relevance, because infection with this virus has so far not been associated with disease in seals. For studies of PhHV-1 infections in seal populations, the testing of paired serum samples for specific antibodies will probably remain the method of choice.

Newborn seals were especially at risk of fatal PhHV-1 infection. Harbour seal pups orphaned before uptake of colostrum may be particularly threatened because the maternal antibodies confering temporary protection are mainly transmitted through the colostrum (Ross *et al.*, 1994). As a result, passive immunization with convalescent seal sera can be useful to prevent serious disease during the first weeks of life in a rehabilitation setting. Active immunization with a non-replicating vaccine could also be considered at an early age, since harbour seals have been shown to be immunocompetent from birth (Ross *et al.*, 1994). In light of their close antigenic relationship with herpesviruses of terrestrial carnivores (Lebich *et al.*, 1994), inactivated or subunit vaccines derived from canid or felid herpesviruses might be of value until a homologous PhHV-1 vaccine become available.

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Section 2.4

Molecular characterization and baculovirus expression of the glycoprotein B of a seal herpesvirus (phocid herpesvirus-1)

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Abstract — A glycoprotein B (gB) gene homologue was identified in a 5.4-kB *Bam*HI genomic fragment of the phocid herpesvirus type-1 (PhHV-1) which represents a widespread and important pathogen of pinnipeds. Sequence analysis revealed a gB-specific open-reading frame comprising 881 amino acids. Phylogenetic analysis gave evidence for a close evolutionary relationship between PhHV-1 and members of the *Varicellovirus* genus of the *α-Herpesvirinae* and canid herpesvirus in particular. In PhHV-1 infected Crandell feline kidney cells gB is expressed as a 113-kDa glycosylated molecule which is proteolytically cleaved into at least two fragments of 67 and 53–59 kDa apparently forming disulfide-linked heterodimers of 140-kDa. Cell surface expression of PhHV-1 gB was confirmed by FACS analysis. Thus, synthesis and processing of the gB protein of PhHV-1 follows a pattern also observed in other *Varicelloviruses*. Since the gB protein of herpesviruses, expressed in the baculovirus system, has been shown to be a suitable target for vaccine design, we used this system for expression of PhHV-1 gB. Recombinant (rec), baculovirus-expressed gB was identified as a 105-kDa glycosylated molecule. Proteolytic cleavage into fragments of 62 and 52 kDa was markedly delayed as compared to wild-type (wt) gB. Wt and rec gB harboured endoglycosidase H (precursor)- as well as N-glycosidase F-sensitive N-glycans (proteolytic fragments). Baculovirus-expressed gB appeared to be antigenically authentic, since it was recognized in radioimmunoprecipitation and immune peroxidase monolayer assays by PhHV-1-neutralizing seal sera and by gB-specific neutralizing murine monoclonal antibodies. Furthermore, PhHV-1 neutralizing antibodies were induced in mice following immunization with baculovirus-expressed gB, indicating its suitability for incorporation in a candidate vaccine for seals.

Introduction

Two species of authentic herpesviruses of pinnipeds, designated phocid herpesvirus type-1 and type-2, (PhHV-1, -2) have been described (Osterhaus *et al.*, 1985; Lebich *et al.*, 1994; Harder *et al.*, 1996a). On the basis of partial sequence analysis, PhHV-1 was tentatively associated with the *Varicellovirus* genus of the *α-Herpesvirinae* whereas PhHV-2 clustered with *γ-Herpesvirinae* (Harder *et al.*, 1996a).

While PhHV-2 infections have not been associated with disease in seals, PhHV-1 represents an important pathogen: Following primary infection, self-limiting upper respiratory disease is most frequently observed. Neonate seals and immunocompromised animals, however, are prone to fatal generalization of the infection (Osterhaus *et al.*, 1985, Frey *et al.*, 1989). Serosurveys indicated high prevalences of PhHV-1 or other closely related herpesviruses among different seal species worldwide (Vedder *et al.*, 1987; Harder *et al.*, 1991a; Stenvers *et al.*, 1992b; Stuen *et al.*, 1994). In seal rehabilitation centers recurrent annual outbreaks of PhHV-1 infections causing losses of pups pose a severe problem and raise demands for a vaccine conferring protection against disease (Osterhaus *et al.*, 1985; Borst *et al.*, 1986; Harder *et al.*, 1997).

Herpesvirus membrane glycoproteins (gps) mediate essential functions during the replication cycle and are important determinants of viral pathogenesis (Spear, 1993). Among several gps which proved to be indispensable for viral replication of α–herpesviruses *in vitro*, the glycoprotein B (gB), in concert with other gps, plays an essential role during the penetration

process of virus into the host cell cytoplasm by a fusion event between the virion envelope and the host cell membrane as well as for virus spread by cell-to-cell fusion (Cai *et al.*, 1988; Pereira, 1994). Representatives of the gB family are expressed by members of all herpesvirus subfamilies and show considerable sequence and structural homologies (Gong *et al.*, 1987; Griffin, 1991; Niikura *et al.*, 1992; Maeda *et al.*, 1992; Ellinger *et al.*, 1993; Goltz *et al.*, 1994; Limbach *et al.*, 1994). Therefore, gB has also become an interesting tool for studies on herpesvirus evolution (Karlin *et al.*, 1994a; McGeoch & Cook, 1994). Furthermore, gB represents a prominent target of both humoral and cellular immune responses particularly against α-herpesvirus infections, and in the mouse model of herpes simplex virus-1 (HSV-1) infection immunity solely to gB has been shown to confer protection against lethal challenge (Ling Chan et al., 1985; Cantin et al., 1987; Blacklaws & Nash, 1990; Ghiasi et al., 1992; Eis-Hubinger et al., 1993). Thus, gB is considered a major constituent in the development of herpesviral subunit vaccines (Inglis, 1995).

Here we present the nucleotide and deduced amino acid sequences of the gene encoding the gB equivalent of PhHV-1 and show that it can be expressed in a baculovirus system as an antigenetically and immunogenically intact protein.

Materials and methods

Virus and cells – Crandell feline kidney cells (CrFK), maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, were used for culturing PhHV-1 (isolate PB84). A modified *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV-BacPAK 6) expressing *β-glucuronidase* (Kitts and Possee, 1993; obtained from Clontech) and other recombinant baculoviruses were grown and assayed in *Spodoptera frugiperda* clone 9 (Sf 9) monolayer cultures according to standard procedures (O'Reilly *et al.*, 1992).

Purification and restriction endonuclease digestion of PhHV-1 DNA – PhHV-1 viral DNA was extracted from mature extracellular virions enriched by ultracentrifugation through a sucrose cushion as described previously (Lebich *et al.*, 1994). Recovery of DNA followed the protocol of Engels *et al.* (1983). Aliquots of approximately 1 μg of viral DNA were endonuclease-digested, separation of fragments was by electrophoresis in 0.7% agarose slab gels at 1.0 V/cm overnight, and fragments were visualized after ethidiumbromide staining.

Southern blot hybridization – Restriction fragments of PhHV-1 DNA were separated in 0.7% agarose gels and blotted using standard procedures (Sambrook *et al.*, 1989). A 1.6-kB PCR amplificate derived from the PhHV-1 (isolate PB84) gB equivalent gene served as a probe in non-radioactive hybridizations (ECL, Amersham). Selection of degenerate primers (upstream: 5'- ACA ACT gTA Tgg TCT gg ; downstream: 5' - ggT AgA AAT TCA CgA TC[C/T] TC) from conserved parts of the canid and felid herpesvirus (CHV and FHV) gB genes as well as PCR conditions were exactly as described previously (Harder *et al.*, 1996a).

Cloning and DNA sequencing – A 5.4-kB *Bam*HI restriction fragment of the PhHV-1 PB84 genome hybridized to the gB specific probe and was cloned into pBluescript SK+ phagemid (Stratagene). Nested sets of deletions were generated from the 5'- end of the insert using an exonuclease

III system (Erase-a-Base^R, Promega). Deletion mutants were cycle-sequenced using dye-dideoxy terminator reactions (PRISM^R, Applied Biosystems) and analyzed on an automatic sequencer (373A, Applied Biosystems). Specific primers (17mers) were then selected to sequence the complement strand. Sequences were finally assembled and further analyzed using the GCG software package, Release 8.0 (Devereux *et al.*, 1984).

Construction of transfer vectors and recombinant baculoviruses – The entire ORF encoding the PhHV-1 gB gene was amplified by PCR from full-length viral DNA. Primers were selected according to the newly established sequences (upstream: 5'- CgC *ggA TCC* AgT TAT gTA TTT AAT TAC; downstream: 5'- Cgg *CTC gAg* TTA gTT ATT AAA CCT CAT C). Italics indicate *Bam*HI and *Xho*I restriction sites enabling directional cloning of the amplificate into baculovirus transfer plasmid pBacPak8 (Clontech) downstream of the polyhedrin promoter (plasmid pBacPBgB). PCR was performed employing a modified polymerase (Expand High Fidelity^R, Boehringer Mannheim) to improve the fidelity of the reaction (Barnes, 1994). Sequence integrity of the *Bam*HI—*Xho*I gB insert was confirmed using specific 17-mer primers. Liposome-mediated co-transfection of Sf 9 insect cells with linearized baculovirus BacPAK 6 DNA (lacking a *Bsu*36 I fragment) and pBacPBgB according to the manufacturer's recommendations (Clontech) yielded recombinants which were plaque-purified. Recombinants were screened for the 2.7-kB gB ORF by PCR using primers Bac 1 (5'-ACC ATC TCg CAA ATA AAT AAg) and Bac 2 (5'-ACA ACg CAC AgA ATC TAg Cg) (Clontech) located in the polyhedrin promoter and terminator regions, respectively. Selected recombinants were further plaque-purified and expanded for expression analysis.

Northern blot hybridizations – The full length 2.7-kB PCR amplificate of the PhHV-1 gB was used as a probe in a non-radioactive Northern blot assay (ECL, Amersham). Total RNA was extracted from mock-infected and PhHV-1 (PB84) infected CrFK cells (24 hr post inoculation, p.i.). Hybridization at 42°C was allowed for 16 hr. Subsequent washing steps were carried out under high stringency conditions.

Immune peroxidase monolayer assay (IPMA) – Semiconfluent Sf 9 cell cultures were infected with recombinant BacPBgB or BacPAK 6, or were mock-infected. CrFK cell cultures, infected with wt PhHV-1 PB84 or mock-infected, were prepared in parallel. After 3 days of incubation confluent monolayers were heat-fixed at 80°C for 2 hr. Serial twofold dilutions in PBS containing 0.05% (v/v) Tween 20 of PhHV-1-specific convalescent seal sera or monoclonal antibodies (MAbs) raised against PhHV-1 (Lebich *et al.*, 1994) were applied to the wells and incubated at 20°C for 2 hr. Wells to which seal sera had been added were processed with protein A-peroxidase conjugates while goat anti-murine IgG peroxidase conjugates were used to detect bound MAbs. 3-amino-9-ethylcarbazole served as a chromogene. Between all incubation steps plates were washed extensively with double-distilled water. Results were read by light microscopy.

Radioimmunoprecipitation assay (RIPA) – CrFK cells either mock-infected or infected with wt PhHV-1 (M.O.I. = 1), and Sf 9 cells infected with BacPBgB or BacPAK 6 (M.O.I. = 4) were metabolically labelled with L – [^{35}S]-methionine/cysteine (Promix, Amersham: 400 μCi per 10^7 cells) for 4 hr at 24 and 48 hr p.i., respectively. A parallel set of cultures was maintained in the presence of tunicamycin (10 μg ml^{-1}), added when cultures were starved for 1 hr in methionine/cysteine-deficient medium. Cell lysis and immunoprecipitations were exactly as described (Barrett *et al.*, 1989). For precipitations MAbs 1.1H3, 2.2F2, and 3.4B3 raised against PhHV-1 by Lebich *et al.*

(1994) were used since these MAbs were found to react to BacPBgB-infected SF 9 cells in IPMA. MAbs 1.1H3 and 2.2F2 were also known to neutralize PhHV-1 isolates. Additionally, precipitations were performed with various pinniped sera obtained from seals of different species with or without virus-neutralizing (VN) antibodies to PhHV-1. For pulse-chase studies CrFK cells infected for 24 hr with wt PhHV-1 and Sf 9 cells infected for 48 hr with BacPBgB cultures were pulsed with $L-[^{35}S]$-methionine/cysteine (400 μCi per 10^6 cells) for 15 min and chased for various times with cold methionine/cysteine. All immunoprecipitates were analyzed in 12.5% SDS-PAGE gels.

Endoglycosidase treatment of immunoprecipitated proteins – Lysates of $[^{35}S]$–radiolabeled CrFK cells infected with PhHV-1 PB84 or Sf 9 cells infected with BacPBgB, were immunoprecipitated with MAb 3.4B3. Extracted proteins were eluted from protein A-Sepharose beads by boiling in Endo H/F buffer (10 mM Tris pH 8.0, 0.5% SDS, 0.1% 2-mercaptoethanol) for 3 min. Digestion with 2 mU endoglycosidase H (cleaves high mannose and hybrid N-linked oligosacharides) or 100 mU N-glycosydase F (removes all N-glycans except those with α-1,3 linked core fucose residues) proceeded at 37° C overnight in the presence of protease inhibitors (Complete^R, Boehringer Mannheim). Digests were analyzed by SDS-PAGE in 12.5% gels.

Immunization of mice – Sf9 cells infected with BacPBgB or wild-type BacPAK6 for 72 hr were washed twice in PBS and resuspended at 10^7 cells per ml PBS. Mice were injected subcutaneously with 0.1 ml (equivalent to 10^6 cells). Injections were repeated 14 and 28 days later. Sera were recovered five days after the final injection.

Herpesvirus neutralization assay – VN antibodies to PhHV-1, CHV, and FHV in seal and mice sera were measured by means of a complement-enhanced virus microneutralization assay as described (Harder *et al.*, 1991a).

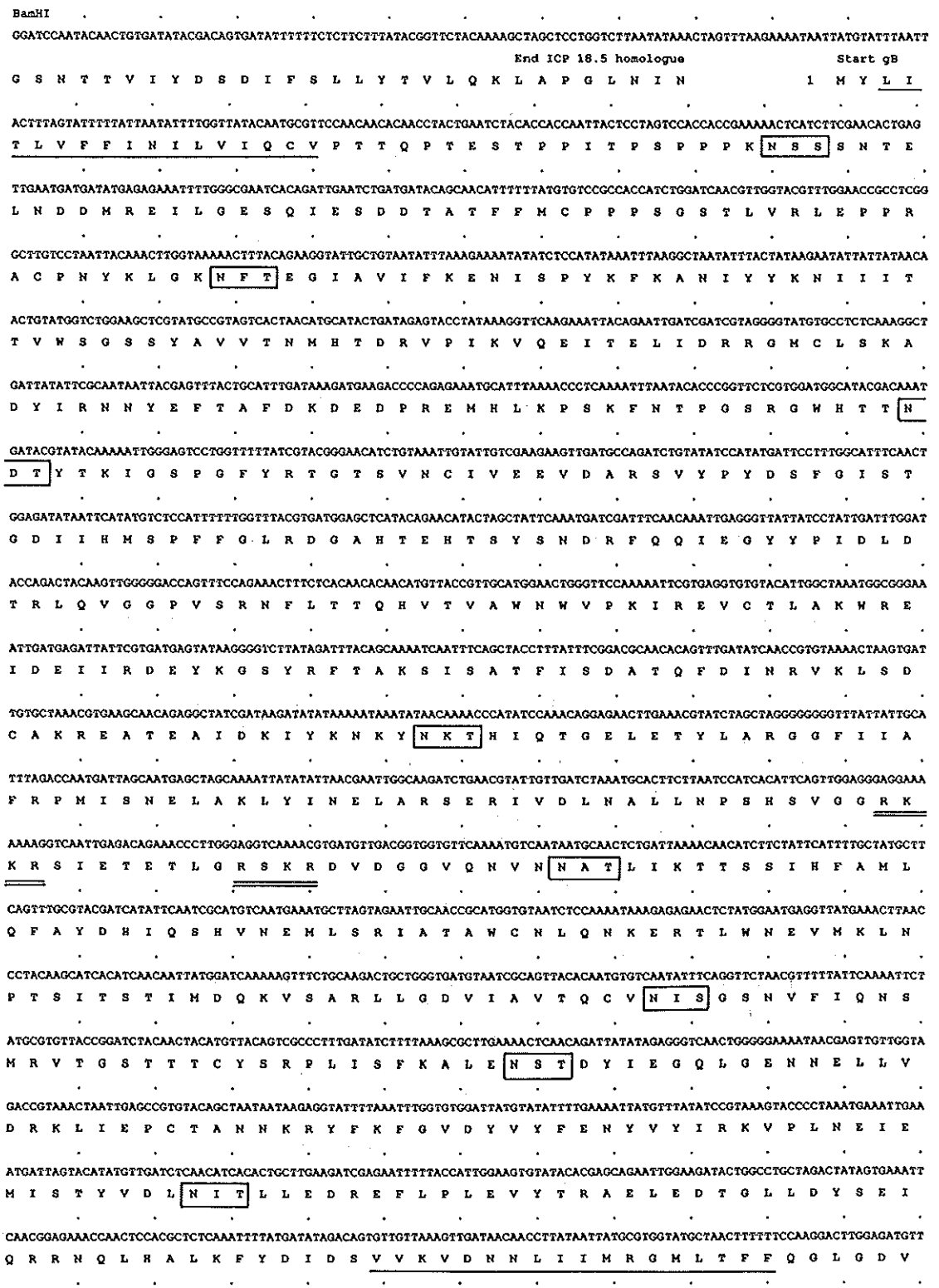
FACS analysis – Native CrFK and Sf 9 cells infected at high M.O.I. with PhHV-1 (20 hr) or BacPBgB (72 hr), respectively, were labeled with MAb 3.4B3 for 1 hr on ice in PBS supplemented with 1% (w/v) BSA (B-PBS). Cell viability exceeded 90% on basis of trypan blue dye exclusion. FITC-labeled goat anti-murine IgG was used as a second antibody. Between all incubation steps cells were washed gently in B-PBS. Mock-infected controls were treated identically. Cells were analyzed in a FACScan (Becton-Dickinson).

GenBank accession – The nucleotide sequence of the PhHV-1 gB gene has been assigned Accession No. Z68147.

Results

Identification and nucleotide sequence analysis of the PhHV-1 gB equivalent gene.

Southern blot hybridizations using a 1.6-kB gB-specific PCR amplificate of the PhHV-1 isolate PB84 as a probe led to the identification of gB-specific sequences in a 5.4-kB *Bam*HI fragment of the PhHV-1 genome (not shown). Fig. 1 presents the nucleotide and deduced amino acid (aa) sequences of open reading frames identified within a 3-kB part of the


BamHI
End ICP 18.5 homologue
Start gB

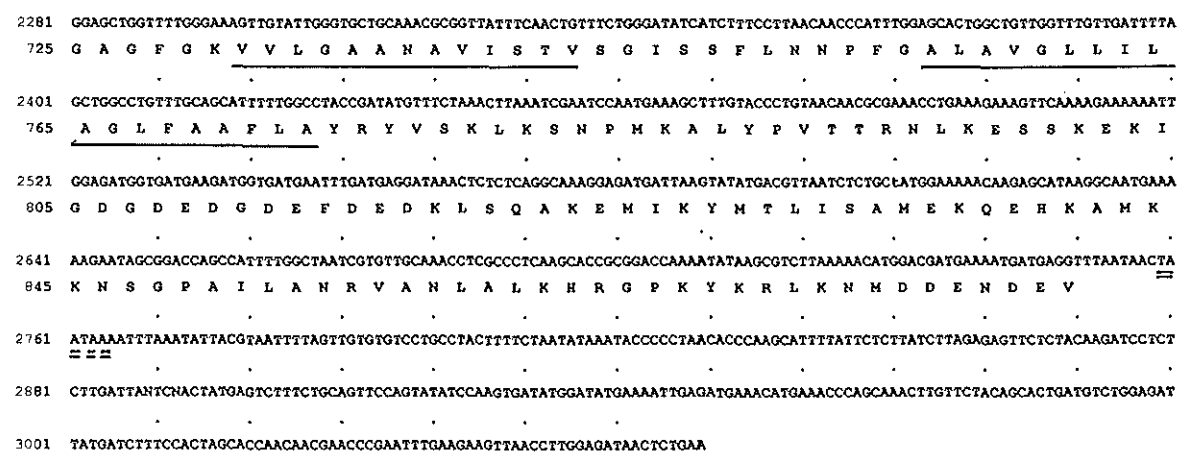


Figure 1. Nucleotide sequence of a part of a 5.4-kB *Bam*HI fragment of PhHV-1 (isolate PB84) and deduced amino acid sequences of open reading frames. A polyadenylation signal is indicated by dashed lines (2760–2765). Putative N-linked glycosylation sites are boxed. Regions of primarily hydrophobic amino acids capable of spanning membranes are underlined. Subtilisin-like serine endoprotease motifs (-R-X-K/R-R-) are double underlined.

respective *Bam*HI fragment which comprises the entire gB gene homologue of PhHV-1 capable of encoding 881 aa. The sequence context according to Kozak's rule (Kozak, 1991) around the putative gB initiation codon (ATT *ATG* T) is well conserved at position -3, but not at +4, which is similar to the gB gene of CHV (Limbach *et al.*, 1994) and other members of the *Varicellovirus* genus. Analysis of sequences upstream of the putative gB ORF revealed homology to members of the UL28 gene family of α-herpesviruses which encodes the ICP18.5 (Pellett *et al.*, 1986).

An overlap of ICP18.5 and gB ORFs as described for several members of the *Varicellovirus* genus including FHV, bovid herpesvirus-1 (BHV-1), pseudorabies virus (PRV) and equine herpesvirus-1 and -4 (EHV-1 and -4) was not found. Thus, PhHV-1 gene arrangements in this region again resemble those of CHV (Limbach *et al.*, 1994). Downstream of the gB ORF several potential initiation codons were located. However, homologies to herpesviral proteins were not found for products translated from any of the putative start codons.

Northern blot analysis gave evidence for at least two gB-specific mRNA species of 3.6 and 2.8 kB in infected CrFK cells at 24 hr p.i. (not shown). Similar gB transcript patterns have been detected in FHV-infected cells (Maeda *et al.*, 1992). A polyadenylation signal (McLauchlan *et al.*, 1985) is present just downstream of the gB termination codon (Fig. 1). Possible gB promoter regions were not identified in this study.

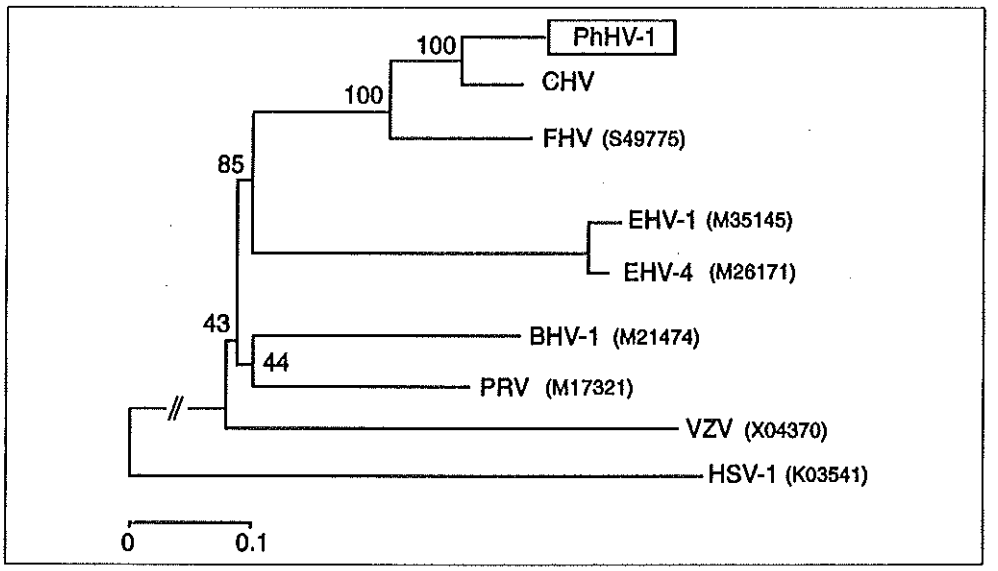


Figure 2. Inferred evolutionary relationships of PhHV-1 based on gB aa sequences. Alignments were computed by 'Pileup' included in the GCG software package (Version 8.0) setting gap creation weights to 3.0 and gap extension weights to 0.1. Further editing included the removal of stretches which could not be unambiguously aligned. Distances were calculated by the Kimura method on the basis of 645 residues and used to construct trees according to the neighbor-joining method. Branches are drawn in proportion to distances as indicated by the bar. Numbers at tree branchings represent bootstrap *P* values after 200 replications. Values $\geq$ 95% indicate a statistically significant separation of the respective cluster from the rest of the tree. Calculations were carried out using the 'Phylip' software package (Felsenstein, 1989). Sequences were extracted from the GenBank database (Accession No. indicated) or from recent publications (CHV, Limbach *et al.*, 1994). VZV, varicella zoster virus.

Predicted PhHV-1 gB amino acid sequence and phylogenetic inferences.

Secondary structure analysis of the deduced amino acid sequence of PhHV-1 gB revealed characteristics of a type-I transmembrane glycoprotein featuring three C-terminal clusters of hydrophobic amino acids (Fig. 1). The most N-terminal stretch of these, however, harbours an arginine residue in a central position and, therefore, is highly unlikely to function as a membrane anchor. Though an N-terminal hydrophobic translocation signal was identified, a possible signal cleavage site was not unambiguously predicted (Nakai & Kaneshisa, 1992) using either the Van Heijne matrix (Van Heijne, 1987) or the method of McGeoch (1985).

Within the putative extracytoplasmic domaine eight potential N-linked glycosylation sites are predicted. A motif of subtilisin-like serine endoproteases (-R-X-K/R-R-; Garten *et al.*,

1994) is found at aa positions 443 - 446 and 455 - 458. Cleavage at or near either of these sites would yield two fragments of approximately 51/49 or 52/48 kDa from a 100-kDa precursor (molecular masses calculated for full-length unglycosylated forms).

To study evolutionary relationships of PhHV-1, deduced aa sequences of gB equivalents of several herpesvirus species were aligned using 'Pileup' (GCG). Stretches which could not be unambiguously aligned were removed and phenetic analysis finally was based on 645 aa residues. Inferences were performed by use of the neighbor-joining principle (NJ) based on distance matrices calculated by Kimura's method and by use of maximum parsimony (PARS) employing a branch and bound approach. The best trees computed by these methods (Phylip software package; Felsenstein, 1989) displayed similar topologies placing PhHV-1 among the members of the *Varicellovirus* genus (NJ: Fig. 2; PARS: not shown). HSV-1, an α-herpesvirus of the *Simplexvirus* genus was chosen as an outgroup. According to the distances calculated and confirmed by the high bootstrap values, CHV was identified as the closest relative of PhHV-1 (Fig. 2).

Synthesis and processing of authentic and baculovirus-expressed PhHV-1 gB.

Baculoviruses carrying the complete gB ORF of PhHV-1 were constructed, and the recombinant BacPBgB was selected for expression studies. Several MAbs (including 1.1H3, 2.2F2, 3.4B3), and all five PhHV-1-neutralizing pinniped sera strongly reacted in IPMA with Sf 9 cells infected by BacPBgB, but not with control cells (BacPAK6 expressing β-glucuronidase, mock-infected Sf 9 cells) (data not shown).

Synthesis and processing of authentic and rec gB were analyzed by RIPA. MAb 3.4B3 specifically coprecipitated proteins of 53-59, 67, and 113 kDa from CrFK cells infected with the parent PhHV-1 isolate (PB84) and proteins of 52, 60-62 and 105 kDa from Sf 9 cells infected with BacPBgB when precipitates were analyzed under reducing conditions (Fig. 3a). Under nonreducing conditions, proteins of approximately 113 and 140 kDa (wt gB) and 105 kDa (rec gB) were depicted (Fig. 3b). MAbs 1.1H3 and 2.2F2 known to neutralize PhHV-1 isolates (Lebich *et al.*, 1994), showed identical reactivities (data not shown). Sera collected from free-ranging pinnipeds reacted to a 105-kDa protein expressed in BacPBgB infected Sf 9 cells only when containing neutralizing antibodies (titers exceeding 1/20 ND_{50}) against PhHV-1 (Fig. 3a). All seal sera tested also reacted weakly to a 135-kDa protein in Sf 9 cells infected with BacPAK 6.

Possible relationships of gB-specific proteins coprecipitated by single MAbs from PhHV-1-infected CrFK cells were investigated in pulse-chase experiments which gave evidence that the 53 to 59, and 67-kDa protein species are generated by proteolytic cleavage from a 113 kDa precursor (Fig. 4a). Cleavage products were demonstrated already after a 15-min chase. However, processing was incomplete since after a 6-hr chase at least 50% of precursor molecules still remained uncleaved. In addition, a 62-kDa protein was weakly coprecipitated from PhHV-1-infected cell lysates by gB-specific MAbs; the intensity of this band decreased during the chase (Fig. 4a).

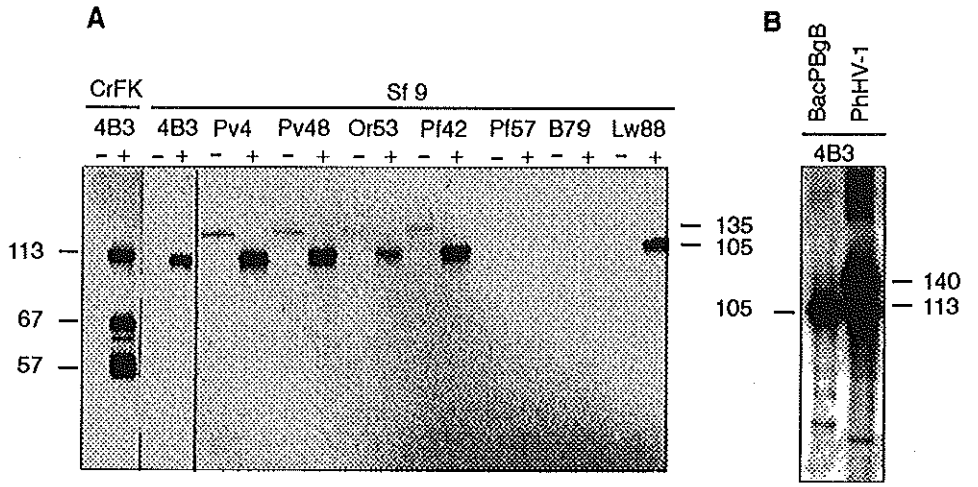


Figure 3. Detection of wt and rec PhHV-1 gB by RIPA. [^{35}S]-methionine/cysteine labeled lysates of CrFK cells infected with PhHV-1 PB84 (+) or mock-infected (-) and Sf 9 cells infected with BacPBgB (+) or BacPAK6 (-) were precipitated using the PhHV-1 specific MAb 3.4B3 (4B3) or various pinniped sera obtained from different species worldwide. Selection of sera was based on presence of neutralizing antibodies to PhHV-1 (titer exceeded 1/20 ND_{50}): Pv4 and Pv48 (common harbour seal, *Phoca vitulina*, US Atlantic coast and Dutch Wadden Sea), Or53 (walrus, *Odobenus rosmarus*, Bering Sea), Pf42 (ribbon seal, *Phoca fasciata*, Bering Sea), Lw88 (Weddell seal, *Leptonychaetes weddellii*, Antarctica). Sera from Pf57 (ribbon seal, *Phoca fasciata*, Bering Sea) and B79 (bearded seal, *Erignathus barbatus*, Norton Sound) had no detectable VN antibodies to PhHV-1. Separation was by SDS-PAGE (12.5% polyacrylamide) under reducing (A) or nonreducing (B) conditions. The apparent M_r of precipitates is indicated.

Pulse-chase studies performed with BacPBgB infected Sf 9 cells likewise revealed proteolytic processing of the 105-kDa baculovirus-expressed PhHV-1 gB precursor (Fig. 4b). Significant amounts of fragments of 52 and 60-62 kDa, however, were present only after 6 hr of chase and even after 24 hr at least 50% remained unprocessed.

To examine glycosylation of wt and rec PhHV-1 gB, immunoprecipitates obtained with MAb 3.4B3 from BacPBgB-infected Sf 9 cells and wt PhHV-1-infected CrFK cells were digested overnight with endoglycosidase H, N-glycosidase F or were left untreated. In parallel, precipitates from PhHV-1-infected CrFK cells labeled in the presence of tunicamycin to shut

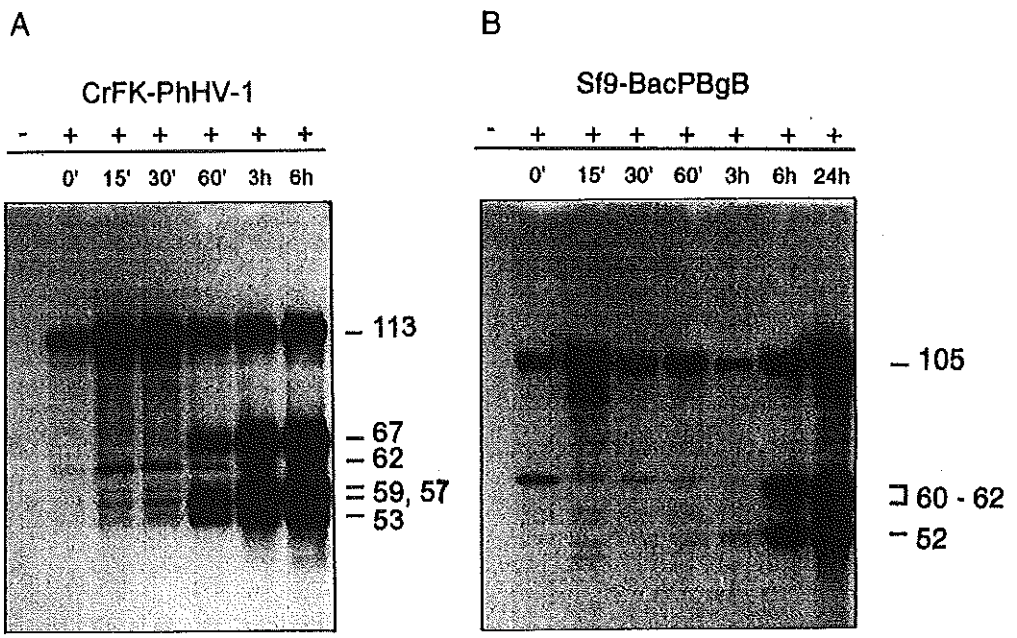


Figure 4: Synthesis and processing of PhHV-1 gB. CrFK cells infected with wt PhHV-1 (A) or Sf 9 cells infected with BacPBgB (B) were pulse-labeled at 24 and 48 hr p.i, respectively, with [^{35}S]-methionine/cysteine at 400 μCi per 10^6 cells for 15 min and then chased for the indicated time periods. Cell lysates were precipitated using MAb PhHV-1 3.4B3. Mock-infected CrFK cells or BacPAK6 infected Sf 9 cells served as controls (–). Separation was by SDS-PAGE (12.5% polyacrylamide) under reducing conditions. The apparent M_r of precipitates are indicated.

off N-linked glycosylation were analyzed. As shown in Fig. 5, proteins of 47/49, 59, and 98 kDa were precipitated from tunicamycin-treated PhHV-1 infected cell lysates. Both wt and rec gB were sensitive to Endo H and Endo F digestion. The shifts observed in molecular masses after digestion were similar in wt and recombinant gB and indicated that the gB precursor molecules were particularly sensitive to Endo H. The fragments, at least, of wt gB were fully sensitive to Endo F, but only partially to Endo H. The smallest wt gB fragment generally migrated as a broad band of 53 – 59 kDa. Following treatment with Endo H,

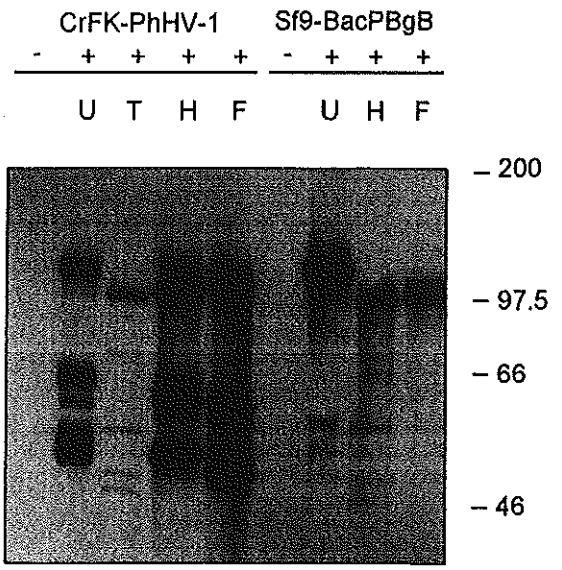


Figure 5: Glycosylation of wt and baculovirus-expressed gB of PhHV-1. PhHV-1-infected CrFK cells or Sf 9 insect cells infected with BacPBgB were metabolically labeled with [^{35}S]-methionine/cysteine at 24 and 48 hr p.i., respectively, for 4 hr. Lysates were reacted with MAb 3.4B3. Precipitates were either left untreated (U) or digested with endoglycosidase H (H) or N-Glycosidase F (F). In addition, precipitates were obtained from infected CrFK cells which were grown in the presence of tunicamycin (T). Mock-infected CrFK cells or BacPAK6-infected Sf 9 cells served as controls (–). Separation was by SDS-PAGE (12.5% polyacrylamide) under reducing conditions. The M_r of marker proteins are indicated.

however, a single protein of approximately 53 kDa was depicted (Fig. 5), indicating that different levels of Endo H-sensitive glycosylation may account for the varying molecular weights.

Cell surface expression of PhHV-1 gB was further examined by FACS analysis of CrFK and Sf 9 cells using MAb 3.4B3. As shown in Fig. 6, expression of the protein was much more pronounced in the CrFK cells than in the Sf 9 cells when analyzed 24 and 48 hr after infection, respectively.

Immunogenicity of recombinant PhHV-1 gB in mice.

Mice immunized with BacPBgB infected Sf 9 cells developed neutralizing antibodies against PhHV-1 with titers ranging from 8 to 22 (reciprocal of ND_{50}). No reactivities in neutralization assays were detected with sera of control mice (immunized with BacPAK6-infected Sf 9 cells).

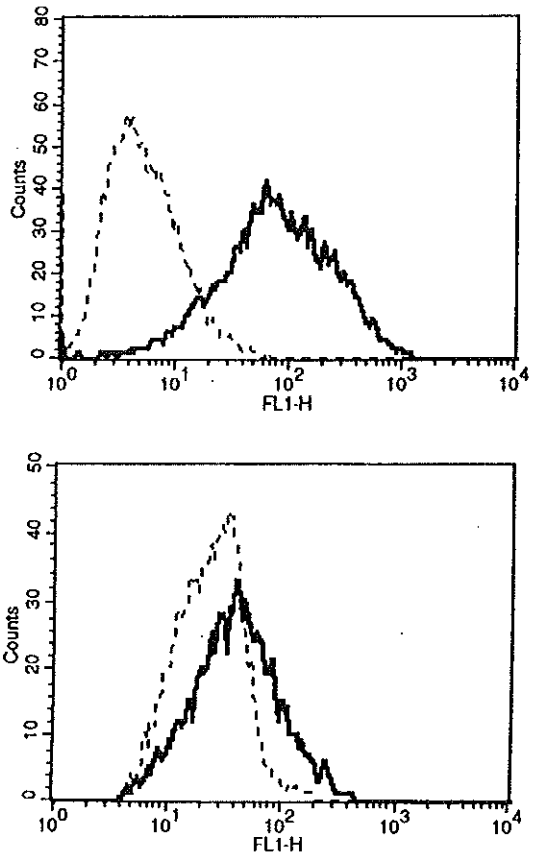


Figure 6. Cell surface expression of PhHV-1 authentic and baculovirus-expressed gB. Viable PhHV-1-infected CrFK cells and BacPBgB infected Sf 9 cells (bold lines) or mock-infected controls (dashed lines) were labeled with the gB-specific MAb 3.4B3. Surface-bound MAbs were detected using FITC-conjugated goat anti-mouse IgG and quantified by analysis in a FACScan.

Discussion

In the present paper we have molecularly characterized the gB of PhHV-1 and expressed it in a baculovirus system. Sequence analysis revealed greatest homology to α-herpesvirus species of the *Varicellovirus* genus which encode a proteolytically cleavable gB. Taxonomic clustering of PhHV-1 was confirmed by phylogenetic inference indicating that the genuine α-herpesviruses of carnivores (FHV, CHV and PhHV-1), like the equine α-herpesviruses, form a separate cluster within this genus. In line with our previous data concerning cross-neutralization and B cell epitope patterns (Osterhaus *et al.*, 1985; Harder *et al.*, 1991a; Lebich *et al.*, 1994), CHV was identified as the closest relative of PhHV-1. Considering recent evidence for a monophyletic origin of pinnipeds from a caniform carnivore ancestor

(Lento *et al.*, 1995), the phylogenetic tree of α-herpesviruses from carnivores overlaps with the phylogeny of their natural hosts. These findings may contribute to the cospeciation hypothesis of α-herpesvirus evolution (McGeoch & Cook, 1994).

Biochemical characterization of PhHV-1 gB confirmed several features predicted from its sequence analysis: In infected CrFK cells the protein is synthesized as a 98-kDa transmembrane molecule which is cotranslationally glycosylated to yield a 113-kDa, Endo H-sensitive product. Further processing involves proteolytic cleavage into at least two, probably disulfide-linked fragments of 53 to 59, and 67 kDa which oligomerize to forms of approximately 140 kDa. PhHV-1 gB cleavage products were already present 15 min after chase in contrast to the gB of PRV which is cleaved after translocation into the Golgi apparatus, requiring at least 60 to 90 min (Whealy *et al.*, 1990). Similar, however, to PRV gB, the gB of PhHV-1 was incompletely processed and at least 50% of the precursor molecules still remained intact after 6 hr of chase. The proteolytic fragments of PhHV-1 gB predominantly contain complex, Endo H-resistant N-glycans. The identity of an additional 62-kDa molecule which was weakly coprecipitated from PhHV-1 infected cells remains to be elucidated. In PhHV-1 infected CrFK cells gB molecules follow the exocytic pathway and are expressed at the cell surface. Synthesis of PhHV-1 gB is, therefore, very similar to other members of the *Varicellovirus* genus, e.g., EHV-1 (Sullivan *et al.*, 1989).

A signal cleavage site as well as the site of cleavage of the fragments of PhHV-1 gB was not unambiguously identified in this study. Most of the endoproteolytically cleavable gB molecules of the different herpesvirus species harbour a single motif (-R-X-K/R-R-) of subtilisin-like serine endoproteases located close to the middle of the primary sequence. The gB of genuine herpesviruses of carnivores, in contrast, contains this motif twice, separated by eight (FHV, PhHV-1) and six (CHV) aa, respectively (Maeda *et al.*, 1992; Spatz & Maes, 1993; Limbach *et al.*, 1994). Whether both sites actually are used and an endoproteolytic fragment is removed during maturation of these gB molecules, as has been suggested for equine herpesvirus-1 gB (Wellington *et al.*, 1996), remains to be investigated.

Synthesis and posttranslational processing of recombinant PhHV-1 gB expressed in baculovirus-infected Sf 9 insect cells resulted in products of slightly lower molecular weight (105, 62, 52 kDa) compared to wt gB, which may be due to differences in N-linked glycosilation in insect cells (Davidson & Castellino, 1990; Kretzschmar *et al.*, 1994). The markedly delayed kinetics of endoproteolytic cleavage of rec gB into at least two fragments in comparison to wt gB cannot be explained by lack of endoproteases with 'R-X-K/R-R' substrate specificity in Sf 9 cells (Du *et al.*, 1994), but probably is at the bottom of the absence of oligomerized forms of rec gB after a 4-hr labeling period. Trafficking of rec gB toward the cell surface seemed to be impaired as only small amounts were detected in the cell membrane of BacPBgB-infected Sf 9 cells in comparison to PhHV-1-infected CrFK cells. Since oligomerization appears to influence correct trafficking of gB molecules via the exocytic pathway (Claesson-Welsh & Spear, 1986; Navarro *et al.*, 1993), the slow formation of the 52/62-kDa fragments of baculovirus-expressed PhHV-1 gB may be related to its low concentration at the Sf 9 cell surface.

Though certain biochemical differences were observed between baculovirus-expressed and

authentic PhHV-1 gB, apparently they did not have a major impact on the antigenicity and immunogenicity of the recombinant protein. Possible protective effects of the PhHV-1 gB specific immunity induced in mice could not be evaluated since a challenge system has not been established. It should be noted, however, that the induced immune responses were comparable to those of mice vaccinated with baculovirus-expressed gB of HSV-1: These mice were shown to be fully protected from lethal intraperitoneal HSV-1 challenge and from induction of latency (Ghiasi *et al.*, 1992). The suitability of baculovirus-expressed gB as a compound of a future PhHV-1 subunit vaccine will be the subject of further studies.

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Section 2.5

Major immunogenic proteins of phocid herpesviruses and their relationships to proteins of canid and felid herpesviruses

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Submitted

Abstract — Immunogenic proteins in cells infected with the α– or the γ–herpesvirus of seals, phocid herpesvirus-1 and -2 (PhHV-1, -2), were examined in radioimmunoprecipitation assays as a further step toward a PhHV-1 vaccine. Using convalescent sera obtained from different seal species or murine monoclonal antibodies (MAbs), at least seven virus-induced glycoproteins (gps) were detected in lysates of PhHV-1 infected CrFK cells. A presumably disulfide-linked complex composed of gps of 59, 67 and 113/120 kDa, expressed on the surface of infected cells, was characterized as a major immunogenic infected cell protein (ICP) of PhHV-1. This gp complex has previously been identified as the proteolytically cleavable glycoprotein B (gB) homologue of PhHV-1 (Harder & Osterhaus, 1997, Virology 227, 343–352). Using MAbs at least three distinct neutralization-relevant epitopes have operationally been mapped on the gB of PhHV-1. Among the ICPs of the antigenically closely related felid and canid herpesvirus, the gB equivalent proved to be the most highly conserved gp. Sera obtained from different seal species from Arctic, Antarctic and European habitats did not precipitate uniform ICP patterns from PhHV-1-infected cell lysates although similar titers of neutralizing antibodies were displayed. Thus, antigenic differences of the α–herpesvirus species prevalent in the different pinniped populations cannot be excluded. PhHV-2 displayed a different ICP pattern, and only limited cross reactivity to PhHV-1 at the protein level was detected which is in line with its previous classification as a distinct species, based on nucleotide sequence analysis, of the γ–herpesvirus lineage. A MAb raised against PhHV-2 and specific for a major gp of 117 kDa, cross reacted with the gB of PhHV-1. The 117-kDa gp could represent the uncleaved PhHV-2 gB homologue.

Introduction

During the last decade, herpesvirus infections have been recognized in a growing number of aquatic mammal species. Herpesvirus infections of pinnipeds were mainly linked to respiratory disease although generalized, fatal herpesvirus infections have been described in free-ranging European harbour seals *(Phoca vitulina)* with concurrent phocid distemper virus infections (Zhang *et al.*, 1989) and as a monoinfection in captive neonate seals (Osterhaus *et al.*, 1985; Borst *et al.*, 1986; Harder *et al.*, 1997a). Herpesvirus isolates from a California sea lion *(Zalophus californianus)* and from several European and American harbour seals showing respiratory or generalized forms of disease have been adapted to cell cultures (Osterhaus *et al.*, 1985; Frey *et al.*, 1989; Lebich *et al.*, 1994; Harder *et al.*, 1996a). On basis of cross neutralization, typing with monoclonal antibodies (MAbs) and nucleotide sequence analysis, two species of seal herpesviruses have been distinguished and were associated with the α–*Herpesvirinae* (phocid herpesvirus-1, PhHV-1) and the γ–herpesvirus lineage (phocid herpesvirus-2, PhHV-2), respectively (Lebich *et al.*, 1994; Harder *et al.*, 1996a).

Serological evidence points toward a moderate prevalence of PhHV-1 or other closely related herpesviruses in various pinniped species, including seals of Arctic and Antarctic habitats (Vedder *et al.*, 1987; Harder *et al.*, 1991a; Stenvers *et al.*, 1992b; Stuen *et al.*, 1994; Zarnke *et al.*, submitted). Disease associated with herpesvirus infection in seals, has so far only been attributed to PhHV-1-like isolates. While firm data on the incidence of PhHV-1 infection in free-ranging pinniped populations are lacking, significant problems arising

from enzootic PhHV-1 outbreaks have been noticed in seal rehabilitation and conservation centers, and raised concern for protection of seals in captivity against PhHV-1 associated disorders (Osterhaus *et al.*, 1985; Harder *et al.*, 1997a). As a first step toward a PhHV-1 subunit vaccine, the glycoprotein B (gB) encoding gene of PhHV-1 has been molecularly cloned, sequenced and successfully expressed in insect cells (Harder & Osterhaus, 1997). In infected mammalian cell cultures, the authentic PhHV-1 gB has been identified as a 113 kDa glycosylated precursor molecule which is proteolytically processed to give two disulfide-linked fragments of 53-59 and 67 kDa (Harder & Osterhaus, 1997). Under non-reducing conditions, heterodimers of 140 kDa are formed.

Here we focuss on the identification of major immunogenic (particularly glycosylated) proteins which are expressed in cells infected with different PhHV-1 isolates and a strain of PhHV-2. At the protein level we confirm and extend previous evidence to distinguish PhHV-1 and -2 as different viral species. Among at least seven glycosilated viral protein species detected in infected cell lysates, the putative PhHV-1 gB is identified as a major immungenic gp which is antigenically highly conserved compared with the gB equivalents of canid and felid herpesvirus (CHV and FHV).

Materials and methods.

Virus and cells – The origin of PhHV-isolates used in this study has been specified elsewhere (Osterhaus *et al.*, 1985; Frey *et al.*, 1989; Lebich *et al.*, 1994). The FHV vaccine strain FVR 605 (Povey, 1975) as well as the PhHV-1 isolates PB84, PB85, 2501/Han88, 2010/Han90, and the PhHV-2 isolate 7848/Han90 were propagated in Crandell Rees feline kidney (CrFK) cells (Crandell *et al.*, 1973). Madin-Darby canine kidney (MDCK) cells (Gaush *et al.*, 1966) were used to grow the CHV isolate 5105/Han89 (Harder *et al.*, 1991a). Experiments were performed using virus material which had undergone at least three plaque purification cycles.

Sera – Titers of herpesvirus-neutralizing (VN) antibodies in sera obtained from seals of different species were determined by a complement-enhanced microneutralization assay as described (Lebich *et al.*, 1994).

Monoclonal antibodies (MAbs) – Production and partial characterization of MAbs raised against PhHV-1 (1.1H3, 2.2F2, 3.1D3, 3.3H5, 3.4G1) and PhHV-2 (2.6E5) has been specified by Lebich *et al.*, 1994. For use in radioimmunoprecipitations, immune-affinity purified preparations of these MAbs were employed.

Definition of operational epitopes– MAbs raised against PhHV-1 were biotinylated according to standard protocols for use in ELISA competition assays. ELISA antigen was produced from PhHV-1-infected CrFK cells which were lysed when at least 80% of the culture showed viral cytopathic alterations. Lysis buffer consisted of 150 mM NaCl, 50 mM TRIS base (pH 8.7) and 20 mM β-octylthioglucopyranoside. Unsoluble residues were pelleted and the supernatant was extensively dialysed against 150 mM NaCl, 50 mM TRIS base (pH 8.7). Antigen-coating of ELISA plates proceeded overnight at 4°C. The optimal antigen dilution in PBS was determined by checker board titrations using unlabeled MAbs. Competetion reactions between biotinylated and unlabeled MAbs

were carried out according to standard protocols. Streptavidin-peroxidase conjugate was used to detect biotinylated MAbs. ABTS served as a chromogene.

Metabolic radiolabeling and immunoprecipitation (RIPA) – MDCK or CrFK cell cultures infected with the various herpesvirus species and showing early stages of viral cytopathic destruction were starved for one hour in methionine (met)- or glucose/pyruvate-deficient medium. Labeling using D-[U-^{14}C]-glucosamine HCL (Amersham $\geq$ 200 mCi/mmol) at 5 μCi/ml or L-[^{35}S]-met (Amersham $\geq$ 1000 Ci/mmol) at 50 μCi/ml proceeded for 16 hr. Labeled cells were lysed on ice in RIPA-buffer (10 mM Tris Base pH 7.8, 150 mM NaCl, 600 mM KCl, 5 mM EDTA, 2%[v/v] Triton X 100, 0.1% [w/v] sodium desoxycholate, and 2.5 mM iodoacetamide), supplemented with 0.5% (w/v) aprotenine. Insoluble residues were removed by centrifugation at 15.000 x G for 15 minutes. RIPAs were carried out following exactly the protocol of Barrett *et al.* (1989). Immunoextracts were separated by SDS-PAGE using 10% or 5 - 15% gradient gels. Fluorographs were scanned and the molecular weight (M_r) of proteins calculated (Ultroscan XL, Gelscan XL, Pharmacia LKB).

Detection of viral gp cell surface expression by fluorescence analysis (FACS) – MDCK or CrFK cell cultures infected with the various herpesvirus species were incubated until the majority of cells had detached due to viral replication. Unfixed, native cells (100.000/reaction; $\geq$ 90 % viable on basis of trypan blue dye exclusion) were incubated with either PhHV-1- or PhHV-2-specific MAbs for one hour on ice. MAb F 3.5, specific for the fusion glycoprotein of canine distemper virus, was used as a negative control. Cells were washed thrice in PBS containing 0.1% of bovine serum albumine and then incubated with goat FITC-labeled anti-murine IgG for one hour. Flowcytometry was carried out on a FACScan (Becton Dickenson) and data were analysed using the Consort software package (Version 3.0).

Table 1. Cross neutralization of herpesviruses of pinnipeds and terrestrial carnivores by seal sera selected for use in RIPA.

		Testviruses[2]			
		PhHV-1	PhHV-2	FHV	CHV
Serum/Year[1]	Host	2557	7848	FVR 605	5101
Pv 26/88	*Ph. vitulina*	230	$\leq$ 2	8	130
Pv 35/92	*Ph. vitulina*	450	3	130	130
Pv 48/90	*Ph. vitulina*	450	45	45	180
Pgr/93	*Ph. groenlandica*	75	$\leq$ 2	23	45
Lw/91	*Lept. weddellii*	64	$\leq$ 2	56	70

[1] Designation and origin of sera: Pv 26 was obtained from a subadult harbour seal after recovery from experimental infection with the PhHV-1 isolate 2501/Han88 (Horvat *et al.*, 1989). Other seal sera were sampled from free-ranging, spontaneously infected individuals: Pv 35 and Pv 48 originated from harbour seals of the North Sea; PhHV-2 7848/Han90 was isolated from lymphocytes of Pv 48 (Lebich *et al.*, 1994). In addition, sera of an Arctic harp seal (Pgr) from the Russian White Sea and of an Antarctic Weddell seal (Lw) were included.

[2] Testviruses employed in complement-enhanced microneutralization assays. Reciprocal titers of neutralizing dose 50% endpoint titrations are indicated.

Results

Major infected cell proteins induced by PhHV-1 and PhHV-2.

Phocid serum PV 26 which was raised against the PhHV-1 isolate 2501 following experimental infection (Horvat *et al.*, 1989), precipitated abundant proteins of 28, 39, 56, 67, 113, 125, 135 and 172 kDa from met-labeled cells (Fig. 1, lanes 3–7). Polypeptides of 41 and 45 kDa were precipitated also from mock-infected CrFK cells (Fig. 1, lane 1). No overt differences of ICP patterns were detected between five European PhHV-1 isolates obtained between 1984 and 1990 from diseased harbour seals ranging in the Dutch and German Wadden sea. For further experiments, therefore isolate 2557/Han88 was used as a PhHV-1 representative. PhHV-2 showed a different ICP pattern with major species of 33, 37, 44, 83, 117 and 135 kDa when precipitated using the autologous phocid serum Pv 48 (Fig. 1, lane 8).

Serum Pv 26, raise against PhHV-1 in a seal, cross reacted efficiently with ICPs induced in FHV and CHV infected cells (not shown), but only marginally with PhHV-2 proteins (Fig. 1, lane 9).

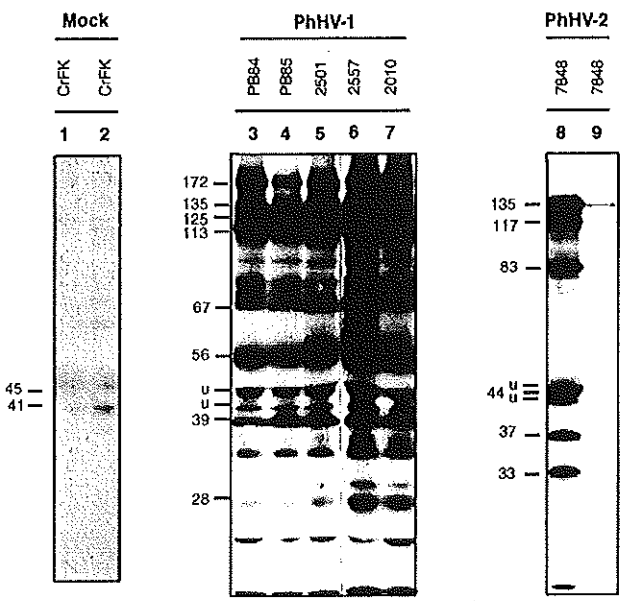


Figure 1. RIPA and SDS-PAGE in 10% polyacrylamide (reducing conditions) of viral proteins in CrFK cells infected with five PhHV-1 isolates (PB84, PB85, 2501, 2557, 2010) or with a PhHV-2 isolate (7848). Lysates were prepared from cells metabolically labeled with *L*-[^{35}S]-methionine. Precipitations were carried out using phocid sera Pv 26 (lanes 1, 3 –7 and 9) or Pv 48 (lanes 2 and 8). See Tab. 1 for origin and properties of these sera. The apparent M_r of major virus-induced proteins is indicated. U – non-specific precipitates.

Antibody specificities to ICPs of PhHV-1 in seal sera from Arctic, Antarctic and European habitats.

Origin and cross neutralizing properties of pinniped sera selected for use in RIPA are presented in Tab. 1. PhHV-1 proteins of 113 and 135 kDa were abundantly precipitated by all pinniped sera which displayed titers of VN antibodies to PhHV-1 (Fig. 2). A serum obtained from a spontaneously infected, free-ranging European harbour seal (Pv 35) which exhibited the highest VN titer, reacted most efficiently to ICPs of 39, 56, 113, 125, 135, and 172 kDa, similar to serum Pv 26. Differences in reactivity patterns were evident when using serum Pgr which had been obtained from an Arctic harp seal (*Phoca groenlandica*) and serum Lw, originating from an Antarctic Weddell seal (*Leptonychaetes weddellii*). While both sera efficiently precipitated the 113 and 135 kDa ICPs, little, if any, reactivity to the 172 kDa protein of PhHV-1 was detected. Although these two sera displayed similar VN titers, serum Lw, in contrast with serum Pgr, did not react to the 28, 39 kDa ICPs and only weakly to ICPs 56, 67 and 125 kDa.

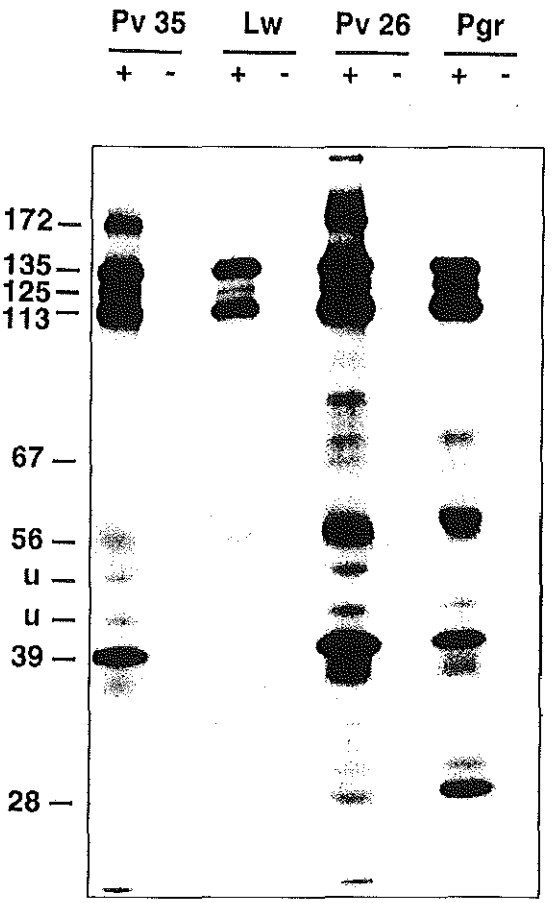


Figure 2. RIPA and SDS-PAGE in 10% polyacrylamide (reducing conditions) of viral proteins in PhHV-1 infected CrFK cells emplyoing sera from seals of different, geographically widely separated populations (Tab. 1). Lysates were prepared from cells metabolically labeled with L-[^{35}S]-methionine which were either mock-infected (-) or infected with PhHV-1 2557/Han88 (+). The apparent M_r of major virus-induced proteins is indicated. U – non-specific precipitates.

Immunogenic glycoproteins expressed by PhHV-1 and PhHV-2.

Employing *D*-[U-^{14}C]-glucosamine HCL for metabolic labeling, at least seven viral induced gps of 59, 67, 75, 83, 113, 120 and 154 kDa were detected in PhHV-1 infected cells by use of serum Pv 26 when separated under reducing conditions (Fig. 3a, lane 1). Under non-reducing conditions, two major gps of 140/160 kDa and a minor species of 83 kDa were demonstrated (Fig. 3a, lane 3). Serum Pv 26 reacted also strongly with a 70 kDa gp of presumably cellular origin in mock-infected CrFK cells which was only depicted when non-reducing conditions were applied for separation (Fig. 3a, lane 4, marked 'c'). In PhHV-1 infected CrFK cells, reactivity against this cellular gp is absent, suggesting that the 70 kDa gp is efficiently down-regulated as a result of the herpesvirus infection (Fig. 3a, lane 3).

Table 2. Properties of gB-specific MAbs raised against PhHV-1 and -2.

MAb[1]	Reactivity in			Epitope[5]
	FACS[2]	PA[3]	RIPA[4]	
PhHV-1 1.1H3	PhHV-1, CHV	100	gp 59-67-113/120	I
PhHV-1 2.2F2	PhHV-1, CHV	100	gp 59-67-113/120	II
PhHV-1 3.1D3	PhHV-1, CHV	≤ 20	gp 59-67-113/120	IIIa
PhHV-1 3.4G1	PhHV-1, CHV, FHV	60-90	gp 59-67-113/120	IIIb
PhHV-1 3.3H5	PhHV-1, CHV, FHV	≤ 20	gp 59-67-113/120	IIIc
PhHV-2 2.6E5	PhHV-2, PhHV-1	≤ 20	gp (88)-117	n.d.

[1] MAbs were raised against the indicated herpesviruses by Lebich *et al.*, 1994.

[2] Cross reactivity of MAbs as measured by FACS analysis.

[3] Percentage of plaque reduction (against 50 pfu) at a MAb concentration of 100 μg/ml (Lebich *et al.*, 1994).

[4] Molecular weights (kDa) of gps precipitated from lysates of cells infected with the homologous virus species.

[5] Determined in competition assays (Fig. 4).

n.d. not determined.

PhHV-1-induced gps were also detected by use of MAbs raised against PhHV-1. Properties of these MAbs are listed in Tab. 2. It should be noted that MAbs 1.1H3, 2.2F2 and 3.4G1, raised against PhHV-1, neutralized the infectivity of PhHV-1 isolates. In addition, all MAbs listed in Tab. 2 reacted to native infected cells in FACS analysis indicating that they are specific for a cell surface-expressed viral protein (FACS data not shown). MAb 1.1H3 co-precipitated major viral gps of 59, 67 and 113 kDa and a minor species of 120 kDa (Fig. 3a, lane 5); non-reducing separation conditions revealed two gps of 140 and 160 kDa (Fig. 3a, lane 7). Identical precipitate patterns were obtained with all other MAbs listed in Tab. 2 (MAb 3.3H5: Fig. 3a, lane 9; other MAbs: data not shown). The 59-67-113 kDa gp complex of PhHV-1 has previously been identified as the gB equivalent (Harder & Osterhaus, 1997). Competition assays showed that the PhHV-1 gB-specific MAbs listed in Tab. 2 defined at least five distinct operational epitopes of which at least three proved to be neutralization-relevant (Fig. 4).

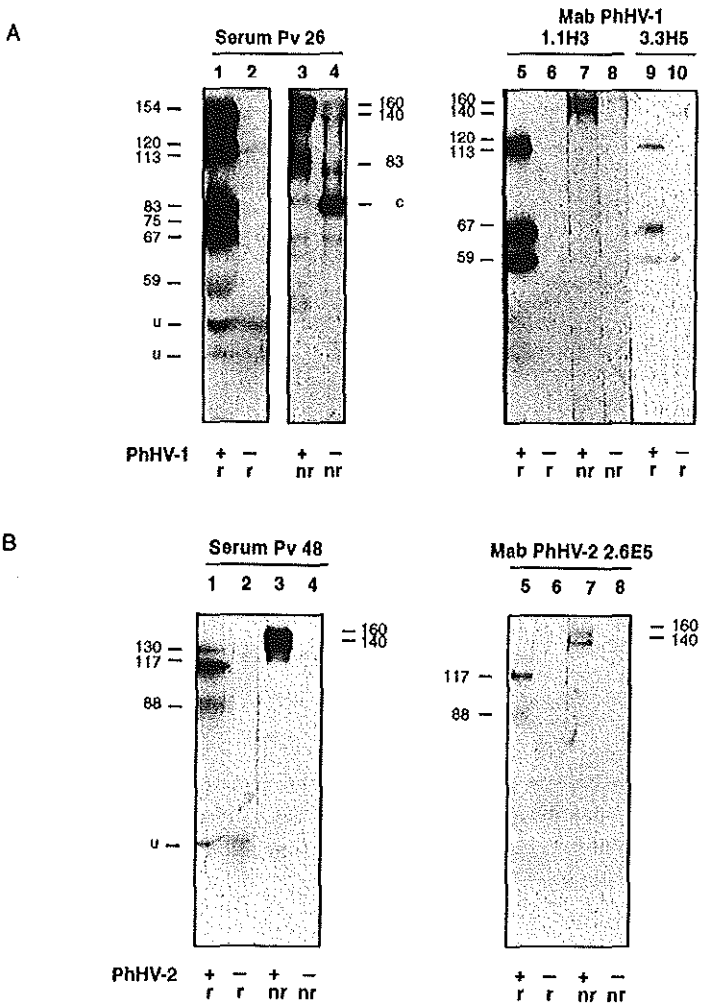


Figure 3. RIPA and SDS-PAGE in 5-15% polyacrylamide gradients of immunogenic gps of PhHV-1 (panel A) and PhHV-2 (panel B). Metabolic labeling of virus-infected (+) or mock-infected (-) CrFK cells was done with *D*-[U-^{14}C]-glucosamine-HCl. Immunoprecipitates were separated under reducing (r) or non-reducing (nr) conditions. The apparent M_r of major virus-induced proteins is indicated. U – non-specific precipitates.

In PhHV-2 infected cells, three gps migrating at 88, 117 and 130 kDa (reducing conditions) or 140/160 kDa (non-reducing conditions) were precipitated using serum Pv 48 (Fig. 3b, lanes 1, 3). MAb 2.6E5, which has been raised against PhHV-2, reacted to a PhHV-2 gp of 117 kDa; in addition, the 88-kDa gp was weakly co-precipitated (Fig. 3b, lane 5). When separated under non-reducing conditions, these precipitates migrated as a double band of 140/160 kDa (Fig. 3b, lane 7).

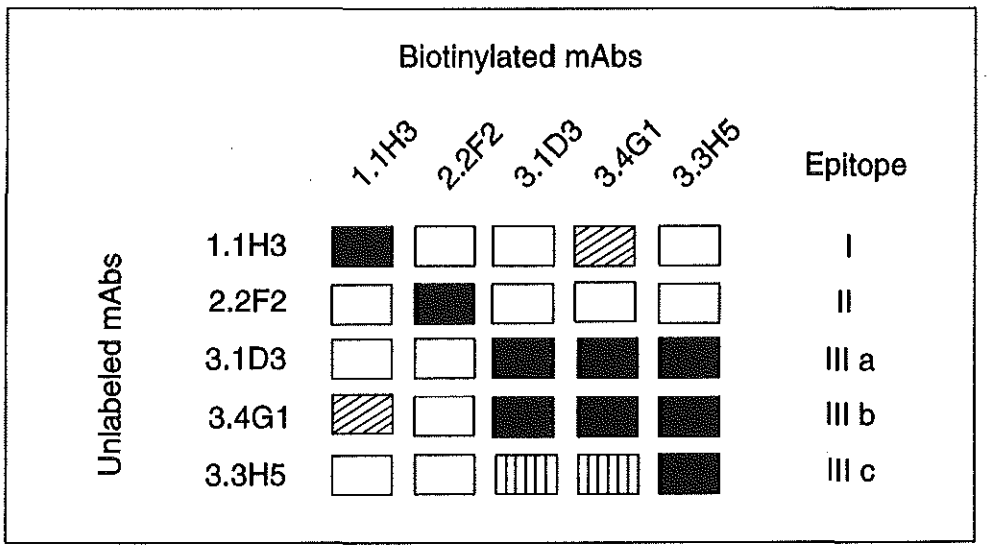


Figure 4. Operational epitope patterns of the glycoprotein B equivalent of PhHV-1. Results were obtained in ELISA competition assays using biotinylated and unlabeled MAbs. ■ Complete competition ($\geq$ 80% inhibition), ▥ partial competition (inhibition 40 – 60%), ▨ enhancement, □ no competition ($\leq$ 20% inhibition).

Conserved B cell epitopes on gps expressed by PhHV-1, CHV and FHV.

B cell epitopes, conserved between gps of PhHV-1, FHV, and CHV, were detected by use of serum Pv 26. Strong cross-reactivity to FHV-induced gps of 62, 67 and 115 kDa and gps of 60, 67, 80, 95 and 115/120 kDa specified by CHV could be demonstrated (Fig. 5a, lanes 4, 5). No gps were cross-precipitated from PhHV-2 infected cells (Fig. 5a, lane 3). Epitope conservation was further explored by use of the broadly cross reactive MAb 3.3H5, raised against the PhHV-1 gB (Tab. 2). This MAb precipitated gB equivalents of 62-67-115/120 kDa and 60-67-115/120 kDa from FHV- and CHV-infected cell lysates (Fig. 5b, lanes 5, 6).

Although no cross-reactivities between gps of PhHV-1 and -2 were detected using serum Pv 26, MAb 2.6E5, specific for a 117 kDa gp of PhHV-2, proved to cross react in FACS with PhHV-1 (Tab. 2). In RIPA, it could be shown that this property is due to reactivity with the gB of PhHV-1 (Fig. 5b, lane 9).

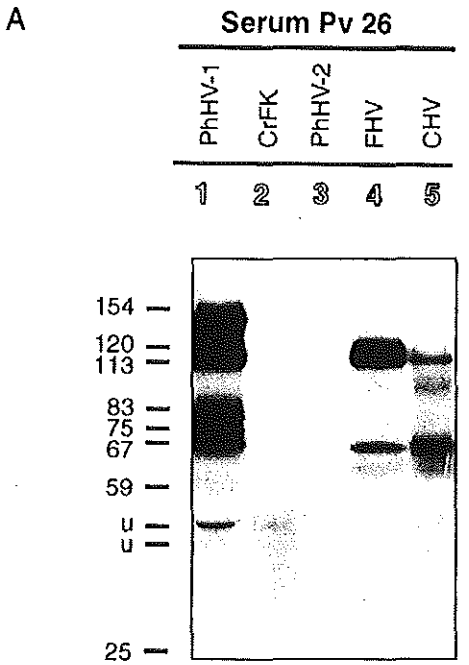
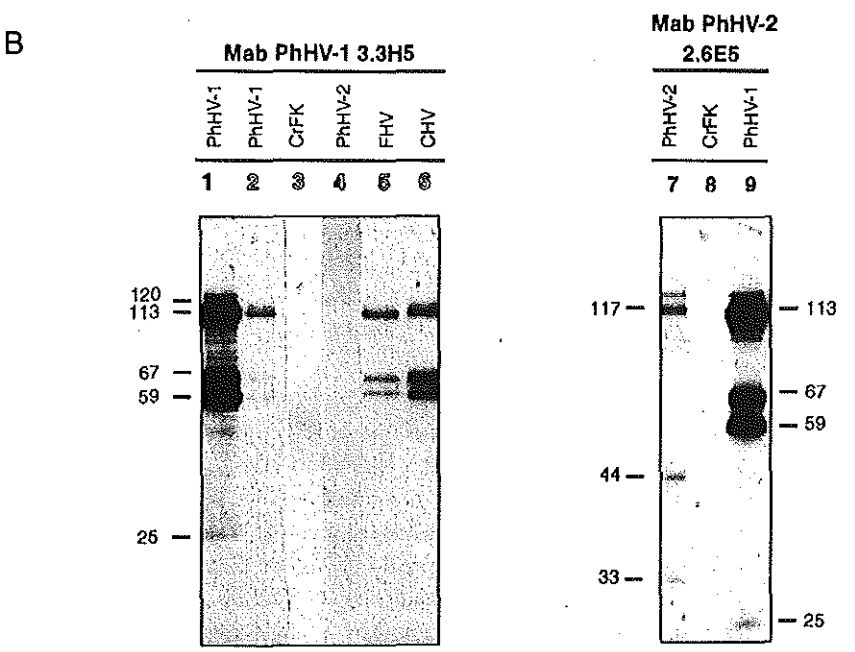


Figure 5. RIPA and SDS-PAGE in 10% polyacrylamide (reducing conditions) of gps antigenically conserved between PhHV, CHV and FHV as detected by polyclonal (panel A) and monoclonal antibodies (panel B). Lysates of mock-infected or virus-infected cells, respectively, were prepared after metabolic labeling with *D*-[U-^{14}C]-glucosamine-HCl (open lane numbers) or with *L*-[^{35}S]-methionine (bold-face lane numbers). U – non-specific precipitates.



Discussion

As a further step towards the development of a homologous PhHV-1 vaccine, we have identified viral proteins in cells infected by PhHV-1 and PhHV-2 which represent major targets of the humoral immune system of naturally infected pinnipeds.

Five PhHV-1 isolates obtained between 1984 - 1990 exhibited similar ICP patterns. In comparison, the PhHV-2 isolate 7848/Han90 differed markedly and cross reactivities between PhHV-1 and PhHV-2 were harshly limited. At the protein level, these results confirm and extend our previous data, based on typing with MAbs and on nucleotide sequence analysis, to classify two distinct species of phocid herpesviruses (Lebich *et al.*, 1994; Harder *et al.*, 1996a).

Sera from European and Arctic seals, naturally infected by PhHV-1, displayed comparable PhHV-1 ICP reactivity patterns. A serum from an Antarctic Weddell seal, however, revealed limited specificity to ICPs of an European PhHV-1 isolate despite similar VN titers compared to an Arctic seal's serum. While a high prevalence of VN antibodies against PhHV-1 has been detected in both Antarctic Weddell seals (Harder *et al.*, 1991a; Stenvers *et al.*, 1992b) and Arctic seal species (Stuen *et al.*, 1995), it cannot be excluded that different, yet closely related, α–herpesvirus species circulate in these populations.

While aiming at the detection of major immunogenic proteins of PhHV-1, we demonstrated more than 20 proteins specifically expressed in PhHV-1-infected CrFK cells of which at least seven were glycosilated. The identification of most of these ICPs, however, remains speculative since up to now only the PhHV-1 gB gene and its products have been molecularly characterized (Harder & Osterhaus, 1997). Among the non-glycosilated ICPs a 135 kDa species was conspicuously precipitated from PhHV-1, PhHV-2, CHV and FHV cell lysates using serum Pv26; by analogy to previously published data, the apparent molecular weight, the relative abundance and the seemingly conserved antigenic structure of this ICP would be compatible with the major capsid protein of herpesviruses (Fargeaud *et al.*, 1984; Manning *et al.*, 1988).

With regard to the development of herpesvirus vaccines, envelope gps attract particular attention because many of them are prominent targets of both the humoral and cellular immune response (Mettenleiter, 1991; Spear, 1993). Especially, the glycoproteins B (gB) and D (gD) of α–herpesvirus species have been shown to elicit high titers of VN antibodies (Para *et al.*, 1985; Backlaws *et al.*, 1990; Ghiasi *et al.*, 1994), and are important targets of cytotoxic T cells (Witmer *et al.*, 1990).

Among the seven gps detected in PhHV-1-infected cells only the 59, 67 and 113/120 kDa species are unambiguously identified, namely as products of the gB gene. We have shown that among the gps expressed by PhHV-1, FHV, and CHV, the gB complex is antigenically highly conserved which has already been predicted by analysis of the deduced amino acid sequences (Harder & Osterhaus, 1997). Cross reactive gB-specific antibodies were also prominent in sera of spontaneously and experimentally infected natural hosts of PhHV-1. Using gB-specific MAbs, at least three distinct neutralization-relevant epitopes were defined. The gB of α–herpesviruses is expected to migrate as a single species or as oligomerized forms

when analysed under non-reducing conditions (Highlander *et al.*, 1991; Maeda *et al.*, 1995). In this study, however, the native gB of PhHV-1 migrated as double band at 140/160 kDa. The identity of the 160 kDa gp, not detected in a previous study of PhHV-1 gB expression (Harder & Osterhaus, 1997) remains to be elucidated. As a possible explanation for this disagreement different labeling periods of 16 hr (this study) versus 4 hr (Harder & Osterhaus, 1997) should be considered. Complex formation involving further viral or cellular proteins during the prolonged labeling period cannot be excluded. In this context, the identification of a 25 kDa protein, coprecipitated by gB-specific MAbs and by polyclonal antibodies (e.g. Fig. 4b, lanes 1 and 9) and likewise not detected in the previous study, will be of special interest. Members of the γ–*Herpesvirinae* subfamily also express gB equivalents which were shown to share a high degree of sequence and structural homology to those of α–herpesviruses (Pellett *et al.*, 1985; Agius *et al.*, 1994; Stewart *et al.*, 1994). MAb 2.6E5 which was raised against the pinniped γ–herpesvirus PhHV-2 cross reacted with the gB of PhHV-1 and precipitated a major 117 kDa gp from PhHV-2 infected cells. It is tempting to speculate that the 117 kDa gp might represent the uncleaved gB equivalent of PhHV-2.

The overall pattern and the molecular weights of further gps preciptated from PhHV-1-infected cell lysates corresponded closely to those described for CHV and FHV, except for a 154 kDa gp which appeared to be unique to PhHV-1. (Fargeaud *et al.*, 1984; Burgener & Maes, 1988; Compton, 1989; Limcumpao *et al.*, 1990; Rota & Maes, 1990; Xuan *et al.*, 1992; Spatz & Maes, 1993, 1994; Maeda *et al.*, 1994, 1995). In addition to the 154 kDa gp, a 83 kDa gp of PhHV-1 was abundantly precipitated by immune seal sera. A putative gD homologue of PhHV-1, by analogy to CHV and FHV (Limbach *et al.*, 1994; Maeda *et al.*, 1994) expected to migrate as a single gp of 55 - 65 kDa, was not identified in this study.

In conclusion, at the protein level we confirmed the existence of two distinct species of phocid herpesviruses. The gB of PhHV-1 was shown to be a major immunogenic gp in the course of natural PhHV-1 infections of pinnipeds and harbours neutralization-relevant epitopes which further justifies considerations to include gB as a component of a future PhHV-1 subunit vaccine. Currently, studies on the identification of further gp-encoding genes of PhHV-1 are in progress.

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Section 2.6

Serologic survey for phocid herpesvirus-1 and phocid herpesvirus-2 in marine mammals from Alaska and Russia, 1978–1994

Randell L. Zarnke, Timm C. Harder, Helma W. Vos, Jay M. ver Hoff and Albert D.M.E. Osterhaus

J. Wildl. Dis., in press

Abstract — Blood samples were collected from 1042 marine mammals off the coast of Alaska and Russia during the period 1978 to 1994. Eight species of pinnipeds were represented. Sera were tested for presence of neutralizing antibodies to both the PB84 isolate of phocid herpesvirus-1 (PhHV-1) and the 7848/Han90 strain of phocid herpesvirus-2 (PhHV-2). Species-specific antibody prevalences ranged from 22% to 77% for PhHV-1 and 17% to 42% for PhHV-2. Species-specific antibody prevalences for PhHV-1 were greater than or equal to prevalences for PhHV-2. For both viruses and each host species, differences in antibody prevalences were not related to: 1. location of capture, or 2. year of collection. Antibody prevalence of PhHV-1 in walruses (*Odobenus rosmarus*) could be quantitatively predicted as a function of age. In sea lions (*Eumetopias jubatus*) and harbour seals (*Phoca vitulina*) females showed a significantly higher PhHV-1 antibody prevalence. The two authentic herpesviruses of pinnipeds have distinct biological properties. The current data indicates that the epizootiology of the two viruses is different, as well. No evidence of herpesvirus-induced mortality has been detected in areas included in this survey. Based on results of this survey, neither PhHV-1 nor PhHV-2 are considered significant mortality factors in mammals which inhabit the marine environment off the coast of Alaska or Russia.

Introduction

Phocid herpesvirus-1 (PhHV-1) is an α–herpesvirus (Harder *et al.*, 1996). It was first identified in 1984 when it caused the deaths of 11 harbour seals (*Phoca vitulina*) pups in a nursery in the Netherlands (Osterhaus *et al.*, 1985). Twelve additional pups which were ill during that epizootic subsequently recovered over the course of a 2-month period (Borst *et al.*, 1986). Mortality due to PhHV-1 infection has only been observed in 1. neonates or 2. seals acutely infected with phocid distemper virus (PDV) or 3. seals which have been otherwise immunocompromised (A.D.M.E. Osterhaus, unpublished).

Clinical signs of PhHV-1 disease included: 1. elevated body temperature, 2. inflammation of the oral mucosa, 3. nasal discharge, 4. coughing, 5. vomiting, 6. diarrhea, 7. anorexia and 8. lethargy (Visser *et al.*, 1991). Duration of illness ranged from 1 to 6 days (Borst *et al.*, 1986). Interstitial pneumonia and necrosis of hepatic parenchyma were the primary histologic lesions. Less significant changes were also observed in kidneys, spleen and lymph nodes (Borst *et al.*, 1986). Experimental evidence indicates that: 1. harbour seals can be infected by means of intranasal instillation of PhHV-1 or direct contact with infected animals, and 2. virus is shed in nasal and ocular discharge from naturally- and experimentally-infected animals (Horvat *et al.*, 1989; Harder *et al.*, 1997). Presumably, natural transmission occurs by means of aerosols or direct contact, as in other α–herpesvirus infections.

Based on serology, approximately 50% out of 49 free-ranging harbour seals which were involved in the 1988 PDV epizootic in the North Atlantic, had been exposed to PhHV-1 (Frey *et al.*, 1989). Despite this high antibody prevalence, PhHV-1 was isolated from only four of 112 harbour seals which had died during the 1988 PDV epizootic (Frey *et al.*, 1989). Serum antibody prevalence for PhHV-1 was 100% in 25 apparently healthy Weddell seals (*Leptonychotes weddellii*) and three apparently healthy crabeater seals (*Lobodon carcinophagus*) from the eastern Weddell Sea (Harder *et al.*, 1991).

A second seal herpesvirus type has been isolated from: 1. a captive California sea lion (*Zalophus californianus*) (Kennedy-Stoskopf *et al.*, 1986), 2. free-ranging adult harbour seals from the North Sea (Lebich *et al.*, 1994), and 3. free-ranging harbour seals from the North Atlantic offshore the United States (Harder *et al.*, 1996). These isolates are collectively known as phocid herpesvirus-2 (PhHV-2). Nucleotide sequence data indicate that PhHV-2 is a γ–herpesvirus (Harder *et al.*, 1996). There is no evidence that PhHV-2 causes clinical disease in pinnipeds. Neutralizing antibodies against PhHV-2 have been detected in a small cohort or harbour seals of the North Sea but not in Weddell seals of Antarctica.

Herpesviruses have been implicated in recent fatal and nonfatal infections of harbour seals in the North Pacific. Twenty-six harbour seals were collected during the investigation of the 1989 *Exxon Valdez* oil spill in Prince William Sound. One male had lesions on the penis and prepuce. Intranuclear inclusion bodies typical of herpesvirus were observed during histologic examination of the lesions (Spraker *et al.*, 1994). The death of a single harbour seals off the coast of Washington State in 1990 was attributed to a herpesvirus. This diagnosis was based upon gross lesions, light microssopy, and electron microscopy (G.M. Zaucha, pers. comm.). Hepatic and adrenal necrosis were found in 12 harbour seal pups which died at a rehabilitation center in Sausalito, California, during 1990. Herpesvirus virions were detected in these necrotic areas by use of electron microscopy (Lowenstine *et al.*, 1992).

Herpesviruses have also been detected in other northern hemisphere marine mammals species including: 1. harbour porpoise (*Phocoena phocoena*) (Kennedy *et al.*, 1992), California sea lion (Kenndy-Stoskopf *et al.*, 1986), and sea otter (*Enhydra lutris*) (Harris *et al.*, 1990).

The purpose of the current project was to determine the serum antibody prevalence of PhHV-1 in marine mammal populations off the coast of Alaska and Russia.

Materials and methods

Collection of samples – Animals were collected by various investigators during studies of marine mammal population biology and ecology. Collection areas included portions of southeastern Alaska, the Gulf of Alaska, eastern and western Bering Sea, Chukchi Sea, and Beaufort Sea (Fig. 1). The following descriptive data were recorded for each animal: 1. sex, 2. date and 3. location. Age data were collected only for walruses (*Odobenus rosmarus*). Age determination for walruses was based on: 1. body conformation, 2. tusk size, and 3. facial characteristics (Fay, 1982). For the purpose of comparison, samples were grouped by year of collection. Year of collection does not neccessarily reflect year of exposure.

Blood was routinely drawn from the extradural vein, caudal gluteal vein, or rear flipper. Samples were allowed to clot and then centrifuged. Serum was transferred to sterile vials. Sera were initially stored at -12°C for several months and then transferred to -40 to -46°C for periods lasting from several months to several years until the time of testing.

Serology – Similar microneutralization assays were used to detect antibodies against both PhHV-1 and PhHV-2 (Osterhaus *et al.*, 1985; Lebich *et al.*, 1994). The PB84 isolate of PhHV-1 was grown in seal kidney cells (Osterhaus *et al.*, 1985). The 7848/Han90 strain of PhHV-2 was propageted in Crandell Rees feline kidney (CrFK) cells (Harder *et al.*, 1996). Both isolates were obtained

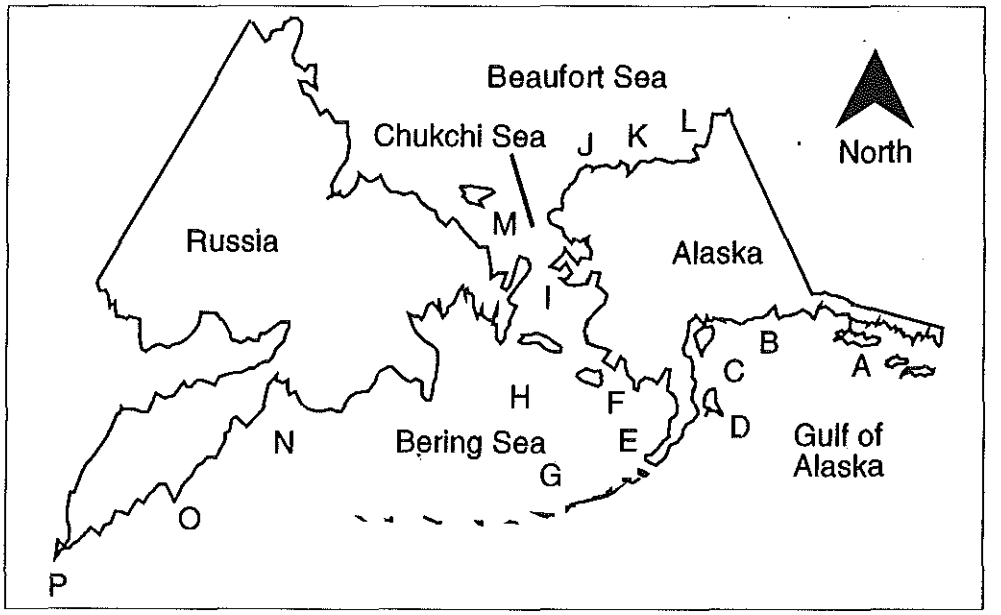

Figure 1. Location of collection sites for marine mammals included in PhHV-1, -2 serologic surveys. A, Southeast Alaska (134°W, 56°N); B, Prince William Sound (147°W, 61°N); C, Cook Inlet (152°W, 60°N); D, Kodiak Island (153°W, 57°N); E, Alaska Peninsula (160°W, 56°N); F, Kuskokwim Bay (163°W, 59°N); G, Pribilof Islands (170°W, 57°N); H, Bering Sea (174°W, 62°N); I, Bering Strait (169°W, 66°N); J, Northwest Alaska (164°W, 70°N); K, Barrow (157°W, 71°N); L, Prudhoe Bay (148°W, 71°N); M, Chukchi Sea (172°W, 69°N); N, Karaginsky Gulf (164°E, 58°N); O, Southeast Kamchatka Peninsula (160°E, 53°N); P, Kuril Islands (150°E, 46°N).

from harbour seals of the North Sea. One hundred mean tissue culture infective doses ($TCID_{50}$ of virus were used in each test. Results were evaluated by development of viral cytopathic effect after 3 days for PhHV-1 and 7 days for PhHV-2.

Under normal test protocol, samples which neutralize PhHV-1 or PhHV-2 at a serum dilution of 1:5 or greater are considered indicative of previous natural exposure to the virus (Osterhaus *et al.*, 1985; Lebich *et al.*, 1994). For the current study a higher threshold dilution of 1:20 was selected. Samples that met or exceeded a titer of 20 will be referred to as positive. All others will be referred to as negative. This change in threshold was implemented in order to reduce the impacts of potential nonspecifc neutralizing or cytotoxic substances on test interpretation. The higher threshold may lead to an underestimation of actual antibody prevalences.

Statistics – For walrus, a generalized linear model with a logit link (McCullagh & Nelder, 1989) was used to determine if there was a significant dependence of serum antibody prevalences on the following variables: 1. age, 2. sex, and 3. year of collection. Antibody response is a binary response variable. Age was treated as a continuous variable. Sex and year of collection were categorical. Geographic location was not included in any logit model for predicting antibody status of walruses.

With one exception, samples were collected from unique locations each year. Therefore, year of collection is confounded with location in the modelling process. All main and interaction effects of these variables were evaluated. During the modelling process, all higher order terms were removed from the model if they did not substantially ($P \geq 0.05$) increase the fit of the model based on the deviance function compared to a Chi-squared value (McCullagh & Nelder, 1989). The SAS statistical software package was used to fit the model with maximum likelihood parameter estimates.

Sex and year-of-collection data for harbour seals, sea lion, and spotted seal were also subjected to the logit modelling process for PhHV-1. Ages were not available for these species. The Chi-square test was used to determine if PhHV-2 antibody prevalence for ribbon seals, spotted seals, or harbour seals was related to sex of the animal.

Results

Serum antibody prevalences for PhHV-1 ranged from 22% to 77% (Tab. 1). Serum antibody prevalences for PhHV-2 ranged from 17% to 42%. In most species, prevalence for PhHV-1 was higher than for PhHV-2. From 11% to 34% of each species had been exposed to both viruses.

For walruses, neither sex nor year of collection had a significant effect on antibody prevalence for PhHV-1 ($P \geq 0.05$). The only factor retained in the model was age ($P = 0.04$). Age is a continuous variable. Therefore, the final model is equivalent to logistic regression. The model predicts the probability of a positive test result as a function of age (Fig. 2):

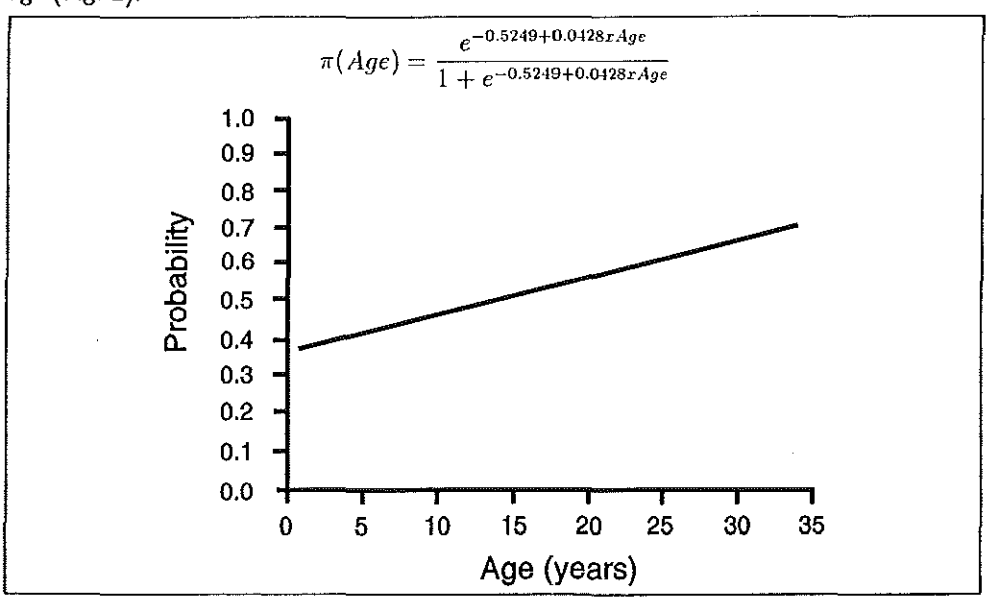

Figure 2. Relationship between walrus (*Odobenus rosmarus*) age and predicted probability of a walrus serum sample exceeding threshold titer (≥ 20) for phocid herpesvirus-1.

For harbour seal, spotted seal, and sea lion the only factor which had an effect on prevalence was sex. Sex is a categorical variable. Therefore, the final model is equivalent to a Chi-square contingency table. Prevalence was higher in females for all three species (103/138 for female harbour seals versus 90/151 for males; 13/25 versus 2/23 for sea lions; and 17/22 versus 19/31 for spotted seals. These sex-specific differences were statistically significant only for harbour seals ($P = 0.007$) and sea lions ($P = 0.001$).

Antibody prevalences of PhHV-2 in all species was not related to: 1. sex, 2. age and 3. year of collection. Antibody prevalence of PhHV-2 for walruses was not a function of age.

Discussion

Cross reactivity is a common problem in serologic surveys. Antibodies produced as a result of exposure to one disease agent may react with a closely-related agent. These circumstances can produce false positive results. Cross neutralization, however, is negligible for most $\alpha-/\gamma-$herpesvirus pairs such as equine herpesviruses-1 and -2, bovid herpesviruses-1 and -4 or varicella zoster virus and Epstein Barr viruses (Plummer *et al.*, 1973; Honess & Watson, 1977; Levings, 1984). In the current study, many sera had titers against either PhHV-1 or PhHV-2. These results concur with a previous study of marine mammals from European waters (Lebich *et al.*, 1994). Therefore, sera with siginifcant titers against either PhHV-1 or PhHV-2 are believed to represent prior natural exposure to the individual virus. Sera with titers against both PhHV-1 and PhHV-2 are believed to represent natural exposure to both viruses.

No phocid herpesvirus isolates are available from the area included in the current survey. Therefore, isolates of European origin were used. Pinnipeds in the current study area may be exposed to viruses which are closely related and yet, antigenically distinct from those found in European waters. Antibody produced in response to these hypothetical viruses may neutralize European strains of PhHV-1 or PhHV-2 less efficiently. These circumstances could yield false negative results. There are minor variations between European isolates of PhHV-1 (Frey *et al.*, 1989). However, there were no significant antigenic differences between isolates of European and United States origin (Harder *et al.*, 1996). Therefore, all positive serologic test results are considered indicative of PhHV-1 and/or PhHV-2 exposure.

Two general taxonomic groups were represented in the survey: 1. phocids – ringed seal (*Pusa hispida*), spotted seal (*Phoca largha*), harbour seals, bearded seal (*Erignathus barbatus*) and ribbon seal (*Histriophoca fasciata*); and 2. odobenids and otariids – walrus, Steller sea lions (*Eumetopias jubatus*) and northern fur seal (*Callorhinus ursinus*). Prevalences for PhHV-1 ranged from 22% to 77% for the phocids and 34% to 54% for the odobenids and otariids. The three highest prevalences were seen in phocid seals. However, there was no clear-cut patterns among or between the two groups. Current antibody prevalences for PhHV-1 are in the range previously reported for hooded seals (*Cytophora cristata*) and harp seals (*Phoca groenlandica*) from the White Sea (Stuen *et al.*, 1994) and for European harbour seals (Frey

Table 1. Serum antiboy prevalences of phocid herpesvirus-1 (PhHV-1) and phocid herpesvirus-2 (PhHV-2) in selected marine mammal species sampled off the coast of Alaska and Russia, 1978 - 1994.

Species	Years of collection	Locations [1] sampled	PhHV-1 [2]	PhHV-2	PhHV-1 and PhHV-2	Negative
Ringed seal *(Pusa hispida)*	1978-1992	H,K,L	10/13 (77%)	2/4	1/4	0/4
Spotted seal *(Phoca largha)*	1978-1993	H,J,K,N,O,P	73/97 (75%)	5/32 (16%)	5/32 (16%)	9/32 (28%)
Harbour seal *(Phoca vitulina)*	1978-1994	A,B,C,D,E,G	161/219 (73%)	52/124 (42%)	42/124 (34%)	19/124 (15%)
Walrus *(Odobenus rosmarus)*	1981-1987	F,I,M	208/384 (54%)	98/341 (29%)	61/341 (18%)	115/341 (34%)
Steller sea lion *(Eumetopias jubatus)*	1978-1993	A,B,C,D,E,G	41/88 (46%)	5/22 (23%)	4/22 (18%)	10/22 (46%)
Bearded seal *(Erignathus barbatus)*	1978-1990	H,K	11/24 (45%)	3/18 (17%)	2/18 (11%)	6/18 (33%)
Northern fur seal *(Callorhinus ursinus)*	1980	G	63/185 (34%)	62/154 (40%)	22/154 (14%)	61/154 (40%)
Ribbon seal *(Histriophoca fasciata)*	1978-1979	H	7/32 (22%)	7/24 (29%)	5/24 (21%)	15/24 (63%)

[1] A, Southeast Alaska; B, Prince William Sound; C, Cook Inlet; D, Kodiak Island; E, Alaska Peninsula; F, Kuskokwim Bay; G, Pribilof Islands; H, Bering Sea; I, Bering Strait; J, Northwest Alaska; K, Barrow; L, Prudhoe Bay; M, Chukchi Sea; N, Karaginsky Gulf; O, Southeast Kamchatka Peninsula; P, Kuril Islands.

[2] Number of samples with significant levels of antibody/total number of samples tested.

et al., 1989). Prevalences for PhHV-2 ranged from 17% to 42% for the phocids and 23% to 40% for the odobenids and otariids.

The highest prevalence value was seen in phocids.

Antibody prevalences for PhHV-1 were generally higher than for PhHV-2 (Tab. 1). Transmission of the highly contagious PhHV-1 is primarily via the respiratory route (Osterhaus *et al.*, 1985; Harder *et al.*, 1997). Routes of transmission for PhHV-2 are unknown. The most effective method of isolating PhHV-2 is via cocultivation of leucocytes (Lebich *et al.*, 1994). Apparently, the virus is highly cell-associated under natural conditions. This conclusion is supported by cell culture under laboratory conditions (Lebich *et al.*, 1994). The cell-associated nature of PhHV-2 makes airborne transmission unlikely. Alternate *routes* of transmission for PhHV-2 may produce lower transmission *rates* than for PhHV-1. These lower rates of transmission may be reflected in the lower antibody prevalences observed in the current survey.

Harbour seals and Steller sea lions live primarily in the subarctic and temperate regions. In contrast, walrus and the remainder of the seal species live primarily in the arctic. There was no discernible pattern of prevalences between these two groups. Apparently, exposure of marine mammals to PhHV-1, PhHV-2, or related herpesviruses has been: 1. common, 2. geographically widespread, and 3. long term.

The logit model quantitatively predicts antibody prevalence of PhHV-1 as a function of age for walruses (Fig. 2). This model apparently reflects: 1. moderate exposure rates at an early age, and 2. continual opportunity for exposure throughout life.

Differential sex-specific mortality rates could contribute to the higher sex-specific antibody prevalence in female harbour seals, spotted seals, and sea lions. If mortality is higher in males than in females, then females would constitute a greater proportion of the older age cohorts. The age effect observed in walrus (Fig. 2) suggests that prevalence would also be higher in older age harbour seals, spotted seals, and sea lions. Unfortunately, age data is not available for these three species.

During the sample collection period, populations of the following species have declined: 1. Steller sea lion (Merrick *et al.*, 1987), 2. northern fur seal (Fowler, 1990), and 3. harbour seal (Pitcher, 1990). No cases with clinical signs of PhHV-1 infection have been reported for either Steller sea lions or northern fur seals. A single case of fatal PhHV-1 infection in a harbour seal was recently reported from the coast of Washington State (G.M. Zaucha, pers. comm.). Thus, there is no apparent relationship between declines of these species and exposure to PhHV-1 or a related herpesvirus.

Based on census data, populations of both walrus (Gilbert, 1989) and polar bear (Amstrup *et al.*, 1986) seem stable. The geographic range of sea otters in Alaskan waters has expanded in recent decades (Rotterman & Simon-Jackson, 1988). Population status for other species is unkown.

Based upon serum antibody prevalences reported here, marine mammals in the waters of Alaska and Russia are commonly exposed to PhHV-1, PhHV-2, or related herpesviruses. Apparently, herpesviruses have become enzootic in these waters. There have been no documented herpesvirus epizootics in these host populations. However, PhHV-1 is capable of

causing morbidity and mortality in nonimmunocompetent seals. Therefore, future outbreaks of clinical disease are possible.

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Chapter 3

Morbilliviruses of seals, dogs and cats

Section 3.1

Intertypic differentiation and detection of intratypic variants among canine and phocid morbillivirus isolates by kinetic neutralization using a novel immunoplaque assay

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Abstract — Intertypic antigenic differences and the intratypic variability of the canine and phocid distemper viruses were examined using a molecular (monoclonal antibodies specific for the H– and F–glycoproteins) and a functional (kinetic neutralization, KN) approach. KN studies were performed by using a novel immunoplaque technique which combined conventional plaque assay and antigen–specific enzyme–immunostaining techniques. Morbillivirus isolates of canine and phocid origin clearly formed two separate groups. Minor antigenic differences were also evident within each cluster. A distemper isolate of mustelid origin was distinguishable from both CDV– and PDV–like prototype viruses by KN studies.

Introduction

Previously undescribed morbilliviruses have been identified as causative agents of a distemper–like disease in several marine mammal species (Osterhaus *et al.*,1988; Kennedy *et al.* 1988a,b; Domingo *et al.*, 1991). Data on viral antigenic and genetic properties suggested that at least isolates derived from harbour seals (*Phoca vitulina*), now referred to as phocid distemper virus (PDV), can be regarded as a new species in the morbillivirus genus. Canine distemper virus (CDV) appears to represent the most closely related of the well-known members of the genus (Mahy *et al.*, 1988; Cosby *et al.*, 1988; Örvell *et al.*, 1990; Haas *et al.*, 1991; Kövamees *et al.*, 1991; Blixenkrone-Møller *et al.*, 1992; Curran *et al.*, 1992). Candidate morbilliviruses obtained from cetacean species such as porpoises (*Phocoena phocoena*) and striped dolphins (*Stenella coeruloalba*) are awaiting a more detailed characterization. The origin of morbilliviruses obtained from marine mammals remained speculative. So far, no isolates linking PDV– and CDV–prototype viruses epizootiologically have been described.

Morbilliviruses form a genus of enveloped negative–strand RNA viruses which are taxonomically placed in the *Paramyxoviridae* family (Pringle, 1992). These viruses are composed of six structural polypeptides of which two are glycosilated and anchored within the lipid envelope (for a review see: Rima, 1983, Morrison, 1988). The H– (haemagglutinin) glycoprotein interacts with membrane receptors of permissive host cells and mediates the attachment between viral and cellular membranes. Following attachment, the F– (fusion) glycoprotein, consisting of two disulfide–linked subunits, initiates the release of the viral nucleocapsid into the host cell cytoplasm by fusion of the attached membranes. The majority of neutralization–relevant epitopes is found on the H–protein whereas the F–protein apparantly carries only a few epitopes which are critical in inactivation of viral infectivity by antibodies (Örvell *et al.* 1985; Sheshberadaran & Norrby, 1986; Malvoisin & Wild, 1990). An immune response directed against both glycoproteins has been shown to play an important role in recovery and protection from morbillivirus infections (Miele & Krakowka, 1983; Varsanyi *et al.*, 1987; De Vries *et al.*, 1988; Rima *et al.*, 1991; Taylor *et al.*, 1991).

A classification of morbilliviruses as PDV– or CDV–like can't be based on host origin of an isolate alone, since PDV– and CDV–like morbilliviruses display a broad and widely overlapping host range both in natural and experimental infection. It is of interest to note that morbillivirus isolates of the CDV–type were recovered from distemper–diseased Baikal seals (*Phoca sibirica*) and, conversely, an outbreak of distemper–like disease in a mink farm was caused by a natural infection with a morbillivirus which was antigenically indistinguishable from a PDV-prototype virus (Visser *et al.*, 1990; Blixenkrone-Møller *et al.*, 1990).

Kinetic neutralization (KN) represents a sensitive method for the differentiation of closely related viruses and intratypic neutralization–variants, respectively (McBride, 1959; Mandel, 1978; Gould & Butler, 1980). The applicability of this technique to members of the morbillivirus genus has formerly met difficulties due to the slow growth and poor plaque–inducing properties of these viruses, especially with regard to wild-type isolates. Nevertheless, Rossiter *et al.* (1989) were able to characterize intratypic neutralization variants of rinderpest virus. In order to circumvent problems encountered with the detection of native plaques Rapp *et al.* (1959) established an immunofluorescence–plaque assay which enabled the detection of measles virus–induced foci by an indirect immunofluorescence technique.

Here we report on the intertypic differentiation and detection of intratypic variants among recent isolates derived from distemper-diseased terrestrial carnivores and harbour seals by a kinetic neutralization technique. The examination of low–passage morbillivirus field isolates was feasible by the development of an immunoplaque assay which combined classical plaque methods and immunocytochemical staining techniques. The isolates were further characterized by typing of antigenic sites of the H– and F–glycoproteins employing a large panel of recently established monoclonal antibodies.

Materials and methods

Cells and virus – Vero cells were maintained in DMEM (Serva Inc.) supplemented with 2% fetal calf serum (FCS). Tab. 1 summarizes the different morbilliviruses (CDV, PDV, MV) used in this study. All strains and field isolates were previously adapted to Vero cells by five to ten serial dilute passages. Stock virus was produced in glass roller bottles (Schott, Germany). A multiplicity of infection (M.O.I) of 0.01 to 0.1 was chosen for the inoculation of subconfluent Vero cell layers in glass roller bottles. Medium was replaced every second day. The cultures were harvested by one freeze/thaw-cycle when at least 80% of the cell layer showed cytopathic changes suggestive of morbillivirus replication. After removal of cellular debris by centrifugation (5000 xG, 15 minutes) the virus suspension was aliquoted and stored at - 80°C.

Sera and monoclonal antibodies – Sera against PDV (isolate 2558/Han88) were raised in two piglets within 31 weeks following intranasal inoculation of virus suspensions (approx. $10^{5.0}$ $TCID_{50}$/animal) at days 1, 131 and 196. Normal porcine serum was obtained from piglets prior to inoculation. The production of a porcine serum against canine distemper virus (strain Rockborn) has been reported previously (Zaghawa *et al.*, 1990).

The production and characterization of murine monoclonal antibodies (MAbs) raised against isolates of PDV (2558/Han88; DK 88 4a) and a vaccine strain of CDV (Convac) was described elsewhere (Örvell *et al.*, 1985; Örvell *et al.*, 1990; Harder *et al.*, 1991b; Blixenkrone–Møller *et al.*, 1992). Exclusively, MAbs specific for the attachment (H) or fusion (F) glycoproteins were selected for this study. The reactivity of each MAb against various morbilliviruses was determined by a peroxidase-linked antibody assay as described below. Selected MAbs were further examined for neutralizing capacities against PDV 2558/Han88 and CDV strain Rockborn (ROC) by a conventional microneutralization assay (see below).

Peroxidase-linked antibody assay (PLA) – Vero cells simultaneously infected with a morbillivirus (M.O.I. 0.01) were grown in microtitre plates (Nunc, Denmark) until a few distinct foci of viral cytopathic alterations within a confluent layer of cells became evident. After being washed once with PBS (diluted to 30% (v/v) in Aq. bidest.) the cell layer was heat-fixed for four hours at 80°C.

Table 1. Origin of viruses.

Strain/Isolate	Origin (Host/cellular source)	Reference
Canine distemper		
'Rockborn' (ROC)[a]	vaccine strain	Rockborn (1958)
'Onderstepoort' (ONST)	vaccine strain	Haig (1948)
5804/Han90	canine/kidney tissue culture	F
1493/Han89	mustelid/lung tissue	F
Phocid distemper		
1970/Han88	phocid/lung tissue	Liess *et al.* (1989b)
2135/Han88	phocid/lung tissue	Liess *et al.* (1989b)
2558/Han88	phocid/leucocytes	Liess *et al.* (1989b)
2953/Han88	phocid/lung tissue	Liess *et al.* (1989b)
Measles		
'More' (att. Enders)[b]	vaccine strain	—

[a] Provided by Dr. G. Rockborn, Biomedical Center, Uppsala, Sweden; [b] provided by Dr. M. Drescher, Hannover Med. School, Hannover, Germany; F – recent isolates obtained from diseased animals.

100 µl of serial twofold dilutions (starting dilution 1 : 20) of each MAb in PBS containing 0.005% Tween 20 (PBST) were added per well and incubated for two hours at room temperature. Appropriately diluted HRPO-conjugated caprine anti-murine IgG was applied to the cavities for another hour at 20°C. 3-amino-9-ethylcarbazole (2 mg dissolved in 300µl dimethylformamid, Sigma Chemicals) diluted in 5 ml of 50 mM acetate buffer (pH 5.0, 0.005% H_2O_2) was used to visualize the reaction. Between each step the plates were washed extensively with PBST. Results were read by light microscopy. The highest dilution of a MAb still mediating an easily visible staining of antigen was recorded as its PLA-titre. For the detection of morbillivirus antigen in neutralization and focus forming assays the MAb PD/TC 4-2 raised against PDV 2558/Han88 was used throughout. This antibody was previously shown to react with a highly conserved epitope located on the nucleocapsid protein (N) of all members of the morbillivirus genus (Harder *et al.*, 1991b).

Focus forming assay (immunoplaque assay) – Vero cells were seeded into Petri dishes (diameter 6 cm, Nunc, Denmark) at a concentration of 1.6×10^6 cells/dish forming a confluent monolayer within 16 hours. Medium was decanted and the cell layer rinsed once with PBS in order to remove loosely attached cells and small aggregates. Virus was diluted in log_{10} steps in DMEM without serum and 1 ml of each dilution was inoculated per petri dish. Virus adsorption was allowed for one hour at 37°C. Subsequently, the inoculum was removed and the cell layer rinsed carefully twice with PBS. Residual fluid was fully drained prior to addition of 7 ml of a semisolid overlay. The overlay medium consisted of a 1% (w/v) solution of Noble agar (Difco Laboratories) in DMEM, supplemented with 5% (v/v) FCS. Following an incubation period of 72 hours the overlay (soaked in PBS for ten minutes) was mechanically removed using a scalpel blade. The cells were briefly rinsed with PBS, air-dried and heat-fixed for 4 hours at 80°C. Staining of morbillivirus antigen was achieved by the peroxidase-linked antibody assay using MAb PD/TC 4-2 as a first layer as described above.

The results were read microscopically at a magnification of 32x. Foci of antigen-positive cells (referred to as immunoplaques) were counted in twenty non-overlapping visual fields along a line

corresponding to the diameter of the Petri dish. The relative concentration of focus-forming units referring to an inoculum of 1 ml was expressed as focus forming units in 20 visual fields per dish (FFUx20VF).

Conventional microneutralization assay – Heat-inactivated sera and selected monoclonal antibodies were assayed for morbillivirus neutralizing properties by a peroxidase-linked microneutralization assay as described in detail by Liess *et al.* (1989a,b). Briefly, 50μl of serial twofold dilutions of test sera or MAbs prepared in quadruplicate were reacted with 50μl of the respective virus suspension adjusted to 100 $TCID_{50}$/50μl for one hour at 37°C. Subsequently, Vero cells were added at a concentration of 6 x 10^3 cells/well. The cultures were maintained for four days until heat-fixation. Morbillivirus antigen was identified immunocytochemically as described above. Wells containing at least a single antigen-bearing cell were regarded 'non-protected'. The neutralizing dose (50% endpoint) was calculated according to Kärber (1931).

Kinetic neutralization assay – Neutralization profiles of several morbillivirus strains against two porcine immune sera raised against PDV 2558/Han88 (serum 254/8) and CDV ROC (serum 253/8) were determined by kinetic neutralization tests (McBride, 1959) employing a focus reduction technique. Additionally, profiles were also measured against the MAb PD/TC 6-2 which recognized an epitope located on the H-protein of PDV isolates (Harder *et al.*, 1991b; Tab. 2).

Virus suspensions were adjusted to 6-8 x 10^3 FFUx20VF/ml. 1.5 ml of this suspension were mixed and incubated at 37°C with an equal volume of diluted immune serum or MAb, respectively. At various times (0, 3, 6, 9, 12, 15, 20, 25, 30, 45, 60 minutes) aliquots of 200 μl were removed, diluted 10^{-1} in chilled PBS and immediately inoculated in duplicate onto accurately prepared Vero cell monolayers in Petri dishes (1 ml/dish). Virus adsorption proceeded for one hour at 37°C. The following steps were carried out essentially as described for the focus-forming assay. Sera and MAb employed in kinetic neutralization were used at a previously determined working dilution capable of reducing the relative concentration of the homologous virus expressed as FFUx20VS by one log_{10} step within 15 minutes (253/8: 1/400, 254/8: 1/40 and MAb 6-2: 1/20). As a control of physical (thermal) inactivation of infectivity kinetic experiments were performed in parallel using a normal porcine serum (morbillivirus-neutralizing titre $ND_50 \leq$ 1:5) at a dilution of 1 : 80 (final).

Arithmetic means of FFUx20VS-values calculated for each virus-serum pair from two separate experiments were subjected to regression and correlation analysis by standard statistical methods. The gradient of each regression curve (b) was recorded as an equivalent of the neutralization constant of the respective virus-serum pair. In order to enable a comparison of the different experiments regression gradients were normalised (N_b) by the formula:

$$N_b = \frac{logb_2}{logb_1}100$$

b_1 - regression gradient of homologous virus-serum pairs

b_2 - regression gradient of heterologous or heterotypic virus-serum pairs.

Results

Production of an immune serum against PDV in swine

Upon repeated intranasal inoculation of the PDV-isolate 2558/Han88 into two piglets morbillivirus-neutralizing antibodies were induced. During the whole period no symptoms attributable to inoculation of PDV were observed. Titres of homologous PDV-neutralizing antibodies

Table 2. Development of morbillivirus-neutralizing antibodies in pigs intranasally inoculated with PDV 2558/Han88.

Animals	Weeks post inoculation								
	1	3	7	13	21	25	28	30	31
254	$\leq 5^a$	40	25	15	520	730	460	920	970
	$\leq 5^b$	≤ 5	23	26	65	53	53	80	65
255	≤ 5	65	30	15	1460	640	530	1600	1400
	≤ 5	≤ 5	26	92	110	80	92	80	110

The animals were inoculated intranasally each with approx. $10^{5.0}$ $TCID_{50}$ in 5 ml DMEM with 2% FCS at days 1, 133 (week 19) and 196 (week 28).
[a] Test virus PDV 2558/Han88 (100 $TCID_{50}$); [b] Test virus CDV Rockborn (100 $TCID_{50}$); titres are expressed as reciprocals of ND_{50}.

assayed by conventional neutralization techniques reached about 1/1000 (ND_{50}). Heterotypic CDV-neutralizing titres were also measurable, but paralleled those against PDV on avarage 1 log_{10} step lower. The development of morbillivirus-specific antibodies is summarized in Tab. 2. In kinetic neutralization studies the serum exhibited a high avidity for its homologous virus (see below).

Development of an immunoplaque assay

The various morbillivirus field isolates obtained from clinical cases of canine, mustelid and phocid distemper did not induce overt viral cpe within 72 hours of incubation in cultures of Vero cells under a semisolid agar overlay. In cultures infected by the vaccine strains CDV Onderstepoort (ONST) and MV MORE occasionally large multinucleated cells became cells became microscopically detectable. Macroscopically visible native plaques could not be observed at all. However, subsequent to immunocytochemical staining solitary redish foci of antigen-bearing cells became evident. In case of CDV ONST and MV MORE these foci reached a diameter of 0.5 to 1.0 mm. Microscopic examination of cultures infected with field isolates revealed small ($\leq$ 0.1 mm) immunoplaques of approximately equal size randomly distributed throughout the culture. Thus, for accuracy of enumeration microscopic evaluation was indispensable. Areas of antigen-positive cells were sharply separated from uninfected cells which facilitated the enumeration of foci considerably. The morphology of immunoplaques varied amongst the isolates. Foci induced by field isolates usually consisted of 5 to 20 infected cells. Immunoplaques induced by MV and CDV ONST were characterized by larger syncytia containing sometimes more than 50 nuclei. Generally, cytoplasmic antigen of all viruses examined was stained with equal intensity. However, mononuclear infected cells gave a more intensive signal compared to the cytoplasm of large multinucleated syncytia. No staining appeared in uninfected cultures. The suitability of the immunoplaque assay for quantitative analysis of virus concentrations was evaluated by repeated titration of various virus suspensions. In all cases a reproducible linear correlation existed between the dilution of the virus suspension and the number of FFUx20VS per dish (data not shown).

Table 3. Clustering of morbilliviruses according to normalized neutralization constants (N_b) measured against two porcine morbillivirus-immune sera and a monoclonal antibody.

Cluster	Porcine sera		MAb
	253/8[a]	254/8[b]	PD/TC 6-2[c]
I ($N_b \leq 70$)	CDV ROC (100) CDV ONST (89) CDV 5804 (73)	PDV 2558 (100) PDV 1970 (97) PDV 2135 (89) PDV 2935 (105)	PDV 2558 (100) PDV 1970 (97) PDV 2135 (115) PDV 2953 (72)
II ($25 \leq N_b \leq 70$)	CDV 1493 (33) PDV 1970 (33) PDV 2135 (40) PDV 2558 (36) PDV 2953 (33) MV MORE (27)	CDV 1493 (29)	
III($N_b \leq 25$)		CDV ROC (10) CDV ONST (14) CDV 5804 (17) MV MORE (11)	CDV 5804 (22) CDV 1493 (20)

[a] raised against CDV Rockborn, ND_{50} against CDV ROC: 1/5000; against PDV 2558: 1/1000,
[b] raised against PDV 2558/Han 88, ND_{50} against PDV 2558: 1/970; against CDV ROC: 1/65,
[c] produced against PDV 2558/Han88 (H-protein-specific), ND_{50} against PDV 2558: 1/1280; against CDV ROC: $\leq 1/5$.

N_b: normalized regression gradient-value (for details of calculation see text). Mean values of each strain/isolate examined are presented in brackets.

Kinetic neutralization

In order to distinguish loss of infectivity due to neutralizing antibodies from physical (thermal) inactivation, kinetic studies were carried out employing a porcine normal serum without morbillivirus–neutralizing titres when checked by the conventional neutralization test ($ND_{50} \leq$ 1:5). The arithmetic mean of regression gradients of all viruses (n=9) was $b_x = 0.96$ ($s = 0.01$) indicating that under the conditions of this study loss of 1 log_{10} of viral infectivity during one hour of incubation at 37°C was expected to be attributable to non–specific physical factors.

The two porcine immune sera and the MAb PD/TC 6–2 showed moderate to high neutralizing titres when tested against the respective homologous virus by conventional neutralization (253–8: 1/5000, testvirus CDV–ROC; 254–8: 1/1000, testvirus PDV 2558; PD/TC 6–2: 1/1280, testvirus PDV 2558). When reacted with the porcine immune sera or MAb PD/TC 6–2, respectively, the neutralization of infectivity in kinetic experiments generally followed a first order exponential function of the type $f_{(t)} = ab^t$. The arithmetic mean of correlation coefficients calculated for 24 regression curves ($r_x = 0.987$) confirmed a strong and functional coherence of the measured values. For easier comparison, the gradient b (representing a measure of the neutralization constant) of each regression curve was normalized (N_b), giving

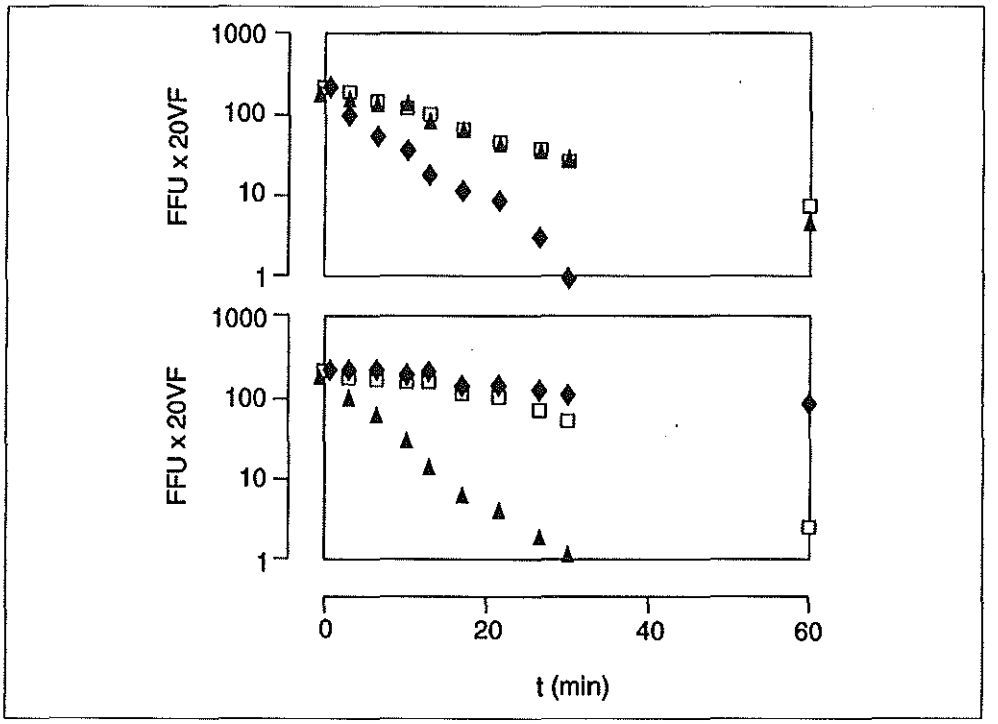

Figure 1: Kinetic neutralization (KN) profiles of different morbilliviruses against porcine immune sera raised against the Rockborn strain of CDV (253/8; top) and the PDV isolate 2558/Han88 (254/8; bottom). KN experiments were performed by using an immunoplaque reduction technique (for methodological details see text). Foci of antigen-positive cells (immunoplaques) were enumerated microscopically. The relative concentration of focus-forming units was expressed as focus-forming units in twenty non-overlapping visual fields (FFUx20VF). ◇ Canine distemper virus (vaccine strain Rockborn); □ mustelid distemper virus (isolate 1493/Han89); △ phocid distemper virus (isolate 2558/Han88).

the gradient of the homologous virus-serum pair a value of 100. Thus, a N_b-value of 30 of a heterologous or heterotypic virus would indicate that the neutralization of infectivity by a certain serum is only 30% complete in relation to the homologous virus within the same time.

According to their N_b-values the viruses could be grouped into three clusters (Tab. 3). Cluster 1 comprised viruses which were rapidly neutralized by the respective serum ($N_b \geq 70$) and therefore regarded to be most closely related to the homologous virus. Cluster 2 contained isolates being moderately neutralized ($25 \leq N_b \leq 70$). Viruses of cluster 3 were not neutralized at all ($N_b \leq 25$) and loss of infectivity was indistinguishable from physical inactivation alone.

A clear intertypic differentiation of distemper isolates of phocid and canine origin as well as measles virus was achieved by both porcine sera and MAb PD/TC 6-2. The mustelid

(ferret) distemper isolate 1493/Han89, however, took an intermediate position and clustered with PDV-isolates when assayed against the porcine anti-CDV serum. On the other hand, 1493 was the only non-phocid isolate which was — though weakly — neutralized by the anti-PDV serum (Fig. 1). Measles virus was weakly neutralized only by the anti-CDV serum. MAb PD/TC 6-2 exclusively neutralized PDV-isolates.

A marked degree of heterogeneity was uncovered among viruses of the N_b-cluster 1 indicating an intratypic variability (Tab. 3). In this respect, the CDV field isolate 5804 was distinguishable from the two CDV-vaccine strains ROC and ONST. A considerable variability was also observed among PDV-isolates when tested against the neutralizing MAb PD/TC 6-2 whereas a more homogeneous picture was obtained with the porcine anti-PDV serum.

Epitope-typing by MAbs directed against morbillivirus glycoproteins

Using a large panel of MAbs previously developed against the H- and F- glycoproteins of CDV and PDV the viruses involved in this study could be grouped into three antigenic clusters, consisting of canine (and mustelid) distemper viruses, distemper virus isolates of phocid origin and, finally, measles virus (Tab. 4a, 4b). The epitope pattern of the F-glycoprotein was considerably conserved among the various viruses and contrasted sharply to the broad intertypic variability of antigenic sites located on the attachment (H) glycoproteins. The majority of MAbs reacted homogeneously within each cluster Trifling intratypic variations could be detected by five MAbs against the H-protein and by one MAb specific for the F-protein (Tab. 4a, 4b). Out of these six MAbs enabling intratypic differentiation only MAB 'H 3.734', raised against the H-protein of the CDV Convac strain, defined an epitope (CDV H 3) which was neutralization-relevant in conventional neutralization assay (data not shown). CDV 1493, isolated from a distemper-diseased ferret, clearly clustered with CDV-like viruses and — in contrast to KN-studies — could not be differentiated from canine distemper virus vaccine strains or a field isolate of canine origin.

Discussion

Since the initial studies of Dulbecco (1952) plaque assay applications are regarded the most precise — though laborious — technique for the quantification of infectious virus *in vitro*. The applicability of this method to members of the morbillivirus genus has been hampered by the poor plaque-inducing properties of morbilliviruses. Vero cells have been reported to be the most suitable cell line for comparative investigations of morbilliviruses (Shishido *et al.*, 1967). However, even well adapted, high-passage vaccine strains required long incubation periods (6 to 21 days) to induce visible native plaques in Vero cell cultures. The majority of plaques did not exceed 1 mm in diameter and, in case of field isolates, were often only of pinpoint size (Gourlay, 1970; Ozawa and Nelson, 1973; Hirayama *et al.*, 1978 and 1986; Albrecht *et al.*, 1981; Cosby *et al.*, 1981).

By combining conventional plaque assay- and enzyme-immunotechniques these problems could be coped with: The immunoplaque assay as used in this study did not only allow to abridge incubation times to three days (which deminishes the risk of secondary plaque-formation) but also allowed the investigation of field isolates. The use of MAb PD/TC 4-2

Table 4a. Reactivity of H-glycoprotein-specific MAbs with different morbilliviruses detected by a peroxidase-linked antibody assay.

MAb	Epi-tope	CDV ROC	CDV ONST	CDV 5804	CDV 1493	PDV 1970	PDV 2135	PDV 2558	PDV 2953	MV MORE
CDV Convac[1]										
1.347	1	+	+	+	+	+	+	+	+	–
2.267	2	+	+	+	+	–	–	–	–	–
3.734	3	+	+	+	+	+	–	+	w	–
3.775	4	+	+	+	+	–	–	–	–	–
4.043	5	+	+	+	+	+	+	+	+	–
4.074	5	+	+	+	+	+	+	+	+	+
4.275	6	(+)	+	(+)	w	(+)	(+)	(+)	(+)	–
4.941	7	+	+	+	+	–	–	–	–	–
PDV DK 88.4a[1]										
1.062 G5	1	–	–	–	–	+	+	+	+	–
1.063 C3	1	–	–	–	–	+	+	+	+	–
1.063 E9	1	–	w	–	–	+	+	+	+	–
1.069 D9	1	+	+	+	+	+	+	+	+	–
1.071 E5	1	–	–	–	–	+	+	+	+	–
1.067 E5	2	(+)	(+)	(+)	(+)	+	+	+	+	–
1.068 F2	3	(+)	(+)	(+)	(+)	+	+	+	+	w
1.070 B5	4	–	–	–	–	+	+	+	+	–
1.072 C4	4	–	–	–	–	+	+	+	+	–
1.085 C4	5	–	w	–	–	+	+	+	+	–
1.122 D11	6	w	+	+	+	+	+	+	+	w
PDV 2558/Han88										
6–2	n.d.	–	–	–	–	+	+	+	+	–
20–2	n.d.	–	–	–	–	+	+	+	+	–
22–2	n.d.	–	–	–	–	+	+	+	+	–
23–2	n.d.	–	–	–	–	+	+	+	+	–

[1] Viral antigen used to raise the specified Mabs.

+ Strong reactivity, PLA-titre $\gg$ 1/1000; (+) moderate reactivity, PLA-titre between 1/100 and 1/1000; w weak reactivity, PLA-titre between 1/20 and 1/100; — no reaction, PLA-titre $\leq$ 1/20; n.d. not determined. Data on epitope specificity are from Örvell *et al.* (1985, 1990) and Blixenkrone-Møller *et al.* (1992).

which reacted with a highly conserved genus-specific epitope for the detection of antigen further opens the possibility to apply this technique to other strains and isolates of of the morbillivirus genus. Essential prerequisites of a regular plaque assay as summarized by Cooper (1961) could be fulfilled: No immunoplaques were detected in non-infected cultures and focus formation was reduced by heat-inactivated specific antisera but not by normal sera, taking into account physical (thermal) inactivation of infectivity. An absolute quantification of focus

Table 4b. Reactivity of F glycoprotein-specific MAbs with different morbilliviruses detected by a peroxidase-linked antibody assay.

MAb	Epi-tope	CDV ROC	CDV ONST	CDV 5804	CDV 1493	PDV 1970	PDV 2135	PDV 2558	PDV 2953	MV MORE
CDV Convac[1]										
3.608	1	+	+	+	+	+	+	+	+	+
3.633	1	+	+	+	+	+	+	+	+	+
5.086	1	+	+	+	+	+	+	+	+	+
3.551	2	+	+	+	+	+	+	+	+	w
3.584	2	+	+	+	+	+	+	+	+	–
3.697	2	+	+	+	+	+	+	+	+	–
4.068	2	+	+	+	+	(+)	w	(+)	w	–
4.985	3	+	+	+	+	–	–	–	–	–
5.148	3	+	+	+	+	+	+	+	+	–
PDV DK 88 4a[1]										
1.062 E2	1	+	+	+	+	+	+	+	+	–
1.068 B2	1	+	+	+	+	+	+	+	+	–
1.067 D2	2	+	+	+	+	+	+	+	+	+
1.073 G10	3	+	+	+	+	+	+	+	+	+
1.092 C3	3	+	+	+	+	+	+	+	+	+

[1] Viral antigen used to raise the specified Mabs.
+ Strong reactivity, PLA-titre $\gg$ 1/1000; (+) moderate reactivity, PLA-titre between 1/100 and 1/1000; w weak reactivity, PLA-titre between 1/20 and 1/100; — no reaction, PLA-titre $\leq$ 1/20. n.d. not determined. Data on epitope specificity are from Örvell *et al.* (1985, 1990) and Blixenkrone-Møller *et al.* (1992).

forming units per dish by microscopic examination was not feasible. Nevertheless, the enumeration of randomly distributed foci in defined parts of the dish gave a consistantly reproducible proportional correlation between virus concentration and induced foci.

The immunoplaque technique described here was used to study intertypic antigenic differences and intratypic variation of distemper isolates of canine, mustelid and phocid origin by kinetic neutralization assays employing polyclonal sera and a neutralizing MAb. A CDV field isolate and two vaccine strains were clearly distinguishable from four PDV isolates by their neutralization profiles. A morbillivirus isolate obtained from a case of distemper in mustelids (ferret), however, took an intermediate position between CDV- and PDV-prototype strains in KN-studies. The characterization of antigenic sites by a large panel of MAbs raised against the H- the H- and and F-glycoproteins of CDV- and PDV-prototype viruses substantiated the interspecies differentiation. The limited degree of reciproke cross neutralization between PDV and CDV isolates as observed by conventional techniques (Liess *et al.*, 1989b; Harder *et al.*, 1991b) and kinetic neutralization as shown in the present study showed that only a few epitopes which are relevant for in vitro-neutralization are shared between the two virus types. This can be interpreted as a result of selection pressure on the H-protein leading to an

optimal adaptation to cellular receptors of different natural host species. The homology of the amino acid composition between the attachment proteins of CDV and PDV was shown to be about 75 % (Kövamees *et al.*, 1991; Curran *et al.*, 1992). These significant differences can not be attributed simply to a recent interspecies transmission of a CDV-like virus to seals.

It may be speculated whether cross neutralization between distantly related morbilliviruses such as CDV and RPV or MV is largely mediated by epitopes of the F-protein. The fusion protein is one of the most conserved morbillivirus proteins and neutralization-relevant epitopes have previously been identified on the MV F-protein by monoclonal antibodies (Malvoisin and Wild, 1990). The demonstration of neutralizing activities in monospecific sera raised against isolated F-proteins, however, proved to be difficult (Norrby & Gollmar, 1975; Varsanyi *et al.*, 1987; Barrett *et al.*, 1989; Taylor *et al.*, 1991).

Antigenic intratypic differences among viruses which were grouped in the same N_b-cluster were evident by a remarkable heterogeneity of neutralization profiles (see Tab. 3, cluster I) which were substantiated by the reaction patterns of five MAbs directed against different epitopes of the H-protein and by one MAb specific for a F-protein epitope. The detection of intratypic variants which differ in biologically active antigenic sites confirm and extend findings findings of a previous study where the same panel of MAbs was applied to a number of different isolates of CDV and PDV (Blixenkrone-Møller *et al.*, 1992). Epitope-typing, however, provided no explanation for the clustering of the mustelid distemper isolate 1493 in KN-studies. Nevertheless, epitopes may be involved which are not detected by any of the MAbs employed. MAbs 'H 1.067 E5' and 'H 1.068 F2' directed against epitopes designated H_2 and H_3 of the PDV-H-protein were previously found not to react with CDV-like strains (Blixenkrone-Møller *et al.*, 1992). In this investigation, however, both MAbs detected corresponding epitopes in all strains of CDV. The observed discrepancy appeared to be due to differences of the test methods employed; in the former study results were obtained by indirect immunofluorescence using acetone-fixed cell-bound antigen. When heat-fixation as carried out routinely in this study was replaced by acetone treatment of morbillivirus-infected cells, both epitopes were found to be lost in CDV-, but not in PDV-strains. Thus, epitopes H_2 and H_3 (PDV-H) are acetone-labile in CDV-like viruses.

In addition to qualitative differences concerning the presence or absence of certain functional epitopes, quantitative variations in the concentration of neutralization-relevant epitopes on the surface of a virus must also be considered when interpreting the occurence of intratypic neutralization variants. It may be assumed that two isolates display H- (and F-) glycoproteins which are identical in regard to their amino acid and glycosilation patterns; however, copies of each of the proteins are incorporated in different numbers into the envelope of each isolate. For a complete neutralization of the isolate which displays lower numbers of glycoprotein copies and thus a lower concentration of functional (neutralization-critical) epitopes, lesser amounts of antibodies are needed. Consequently, this isolate will be more rapidly neutralized by equimolar amounts of antibodies compared to isolate with higher concentrations of neutralization-relevant epitopes. This phenomenon has recently been demonstrated among cell culture-variants of the Edmonston strain of MV where considerably higher titres of neutralizing antibodies in specific sera were measurable when a high-passaged, plaque-purified variant was used and compared to low-passage virus material (Albrecht *et al.*, 1981). Differences concerning the density of otherwise identical neutralization-critical epitopes could

contribute to the observed heterogeneity among viruses grouped within the N_b–cluster I of this study. Furthermore, none of the isolates used were plaque-purified and therefore are likely to represent genetically and probably also phenotypically inhomogeneous populations.

At present, possible *in vivo*–effects of the occurence of intratypic neutralization variants of distemper viruses can not be assessed. The fact, however, that two recent CDV isolates appeared to be distinguishable from two commonly used vaccine strains demands further investigations of a larger number of recent distemper field isolates. The immunoplaque assay proved to be a useful alternative in quantitative virological investigations in virus–cell systems when cell culture properties of the viruses under study interfere with the performance of regular plaque assays which may be encountered in studies of e.g. non–cytopathogenic pestiviruses (Howard *et al.*, 1987) or retroviruses (Fevereiro *et al.*, 1991).

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Section 3.2

Phylogenetic evidence of canine distemper virus in Serengeti's lions

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Abstract — Recently an epizootic, reported to be due to a morbillivirus infection, affected the lion population of the Tanzanian Serengeti National Park. A morbillivirus phosphoprotein (*P*) gene fragment was amplified by PCR from tissue samples of several affected lions. Sequencing of the amplificates and subsequent phylogenetic analyses revealed that a wild-type strain of canine distemper morbillivirus (CDV) was involved. Vaccination of the local domestic dog population with proven safe CDV vaccines is proposed.

Recently, a considerable proportion of the lion population of the Tanzanian Serengeti National Park, has succumbed to a disease that closely resembles distemper in dogs and seals, which are caused by the morbilliviruses canine distemper virus (CDV) and phocine distemper virus (PDV), respectively (Appel & Gillespie, 1972; Osterhaus *et al.*, 1988). On basis of the clinical symptoms observed, a morbillivirus aetiology was suspected and subsequently confirmed by demonstrating immunofluorescence with morbillivirus specific antisera in organs of dead lions, and by showing the development of morbillivirus specific serum antibodies in affected animals (cited after Morell, 1994). Details of the distemper outbreak in lions in the Serengeti are being reported separately (Roelke *et al.*, 1996). Until recently, felids were not considered natural hosts of morbilliviruses. However, in 1991 and 1992 infections with CDV-like morbilliviruses caused enzootics amongst large felids in zoos in the USA (Appel *et al.*, 1994). To acquire insight in the possible origin of the viruses involved in these outbreaks in large felids, we have conducted a phylogenetic analysis of the viruses involved. To this end we have sequenced and compared a *P* gene fragment of the respective morbilliviruses, which was previously shown to be informative for determining phylogenetic relationships of the presently known members of the genus *Morbillivirus* (Barrett *et al.*, 1993).

Brain and lymphoid tissues obtained from five lions which had died in the Serengeti National Park during the outbreak (kindly provided by Dr. Hans Lutz, Dept. of Internal Veterinary Medicine, University of Zurich on behalf of the Messerli Foundation of Zurich, Switzerland) were processed for reverse transcription of total RNA with random hexamer primers and subsequent PCR amplification of the relevant *P* gene fragment using the suitable primer set (Barrett *et al.*, 1993). The amplificates were identified as *P* gene derived by Southern blot analysis using a CDV *P* gene fragment. In the tissues of four out of the five lions, the relevant *P* gene fragment was detected (not shown). *P* gene fragments derived from brain material of two lions were cloned and sequenced, using standard techniques (Murphy & Kavanagh, 1988). The sequences were identical and were subsequently compared to corresponding recently determined *P* gene fragments of other morbilliviruses. These included CDV vaccine strains (Onderstepoort and Bussell), recent CDV isolates from a dog in Europe and from a seal in Siberia (PDV-2) as well as recent isolates from a grey fox (A92-9A) and a captive black leopard (A92-6) from the USA, PDV, measles virus (MV), rinderpestvirus (RPV) and two cetacean morbilliviruses (DMV and PMV, for review see Barrett *et al.*, 1993; Visser *et al.*, 1993a). Sequence alignments of the *P* gene fragment of a Vero cell adapted CDV isolate from a captive black leopard (A92-6; Appel *et al.*, 1994) and a PCR amplificate obtained from brain material of a Serengeti lion (94-28/PLE) are shown in Fig. 1.

```
                .         .         .         .         .
Onst        AAGAGGTTAAGGGAATCGAAGATGCTGACAGTCTCGTGGTACCTGCAGGC   50
A92-9a      ..................................................
A92-6       ..................................................
94-28/PLE   ................C.............................A..

                .         .         .         .         .
Onst        ACTGTCGGTAATCGAGGATTCGAGAGAGGAGAAGGAAGCCTTGATGATAG  100
A92-9a      G.....A.............G.........................
A92-6       G.....A.........A.............................
94-28/PLE   G.....A.............................A..........

                .         .         .         .         .
Onst        CACTGAGGATTCTGGCGAAGATTATTCCGAAGGAAATGCTTCATCTAACT  150
A92-9a      ............................g.................
A92-6       ............................g.................
94-28/PLE   ............................g.................

                .         .         .         .         .
Onst        GGGGATATTCTTTCGGCCTTAAACCGGACAGAGCAGCTGATGTGAGCATG  200
A92-9a      .......................a..........................
A92-6       .......................a..........................
94-28/PLE   .......................a..........................

                .         .         .         .         .
Onst        CTGATGGAAGAGGAATTAAGTGCTCTACTCAGGACAAGCAGAAATGTAGG  250
A92-9a      .............g..g.................A.............
A92-6       .................g........g....A..................
94-28/PLE   .................g........g.......................

                .         .         .         .         .
Onst        GATTCAGAAAAGGGATGGGAAGACTCTGCAGTTCCCACATAATCCCGAAG  300
A92-9a      ...a.....................................c.....a....
A92-6       ...a.....................................c.....a....
94-28/PLE   .....................................c.....a....

                .         .         .         .         .
Onst        GTAAGACAAGGGATCCGGAGTGTGGATCCATTAAAAAGGGCACAGAAGAG  350
A92-9a      ........G.....................................G....
A92-6       ........G.........G.....................t....G....
94-28/PLE   ........G................A..................G....

                .         .         .         .
Onst        AGGTCAGTCTCACATGGAATGGGGATAGTTGCTGGATC   388
A92-9a      .......C..............................
A92-6       .......C..............................
94-28/PLE   ....T..C..............................
```

Figure 1. Sequence alignment generated by 'Pileup' of the GCG software package (version 8.0) of a 388 bp P gene fragment (sequences of PCR primers are excluded) of the Onderstepoort (*Onst*) vaccine strain of canine distemper virus (GenBank acc. no. X51869), Vero cell-adapted CDV isolates of a black leopard (A92-6) from a North American zoo and of a grey fox (A92-9A) from the USA as well as a PCR amplificate obtained from brain material of a free-ranging lion of the Tanzanian Serengeti Nationalpark (94-28/PLE). Identical nucleotides are shown as dots. Non-synonymous mutations to the ONST sequence are shown in upper case.

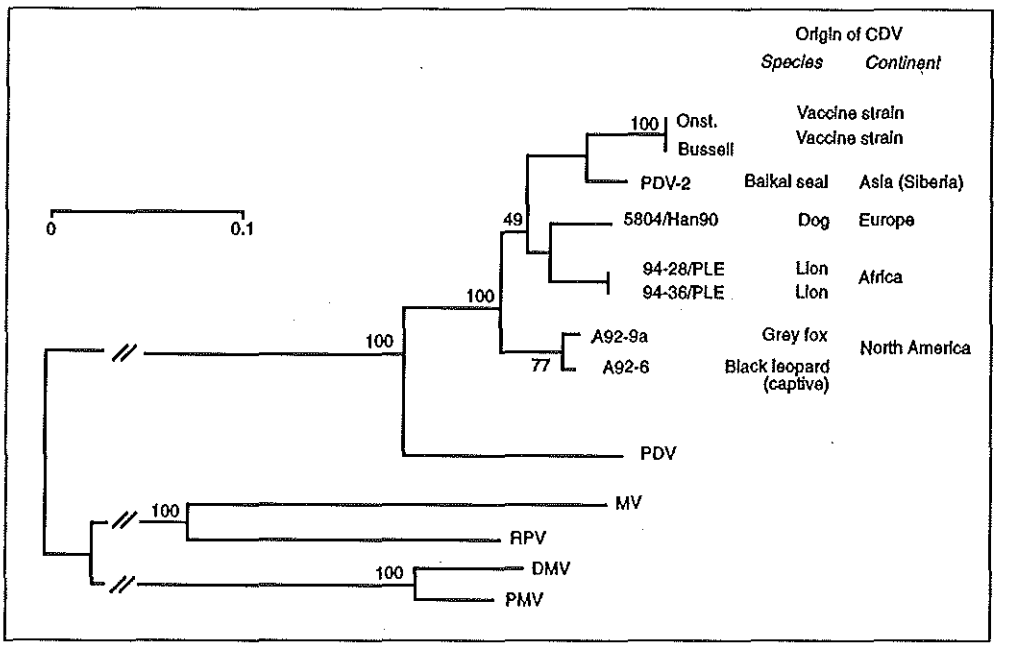

Figure 2. Phylogenetic tree, generated by the neighbor joining method (Saitou & Nei, 1987), of morbilliviruses based on analysis of a 388 bp P gene fragment (excluding sequences of primers used for PCR). The robustness of the tree was statistically analysed by the bootstrap method employing 2000 replications (Felsenstein, 1985; Hedges, 1992). Values of $\geq$ 95 indicate statistic significance that the respective cluster is separated from the rest of the tree. Maximum parsimony analysis of the data gave similar results (not shown). Branch lengths are proportional to the genetic distances which were calculated by the Kimura-2-parameter method (Kimura, 1980). Sequences were aligned by using 'Pileup' of the GCG software package (release 8.0) setting gap weights to 3.0 and gap length weights to 0.1. Within the compared fragment no gaps were observed. MV – measles virus (M89920); RPV – rinderpest virus (X68311); DMV, PMV – dolphin and porpoise morbilliviruses; PDV – phocid distemper virus (European harbour seal morbillivirus, D10371); ONST (X51869), Bussell – canine distemper virus (CDV) vaccine strains; 5804/Han90 – CDV field isolate obtained from a dog in Germany; PDV-2 – phocid distemper virus type-2 (Baikal seal morbillivirus); A92-6 – CDV isolate of a captive black leopard from the USA; A92-9A – CDV isolate of a grey fox from the USA; Serengeti lions 94-28/PLE and 94-36/PLE – sequence of PCR amplificates of the *P* gene fragment generated from brain material: three, respectively two, clones were sequenced, all showed an identical sequence. Sequences were either extracted from the GenBank data base (acc. no given in brackets) or from recent publications (Barrett *et al.*, 1993; Mamaev *et al.*, 1995, 1996).

From the phylogenetic tree constructed on basis of these data, it may be concluded that the morbillivirus that caused the epizootic amongst the Serengeti lions belongs to the CDV cluster of morbilliviruses (Fig. 2). The data also indicate that the viruses which caused the recent outbreaks amongst large felids in the U.S.A. and in Africa are no new or separate *felid* morbilliviruses but rather viruses circulating in domestic and wild carnivores that from time to time also cross species barriers into feline hosts. Similarly, evidence for CDV and PDV infections in pinniped species and for DMV and PMV infections in cetacean species was recently reported (Osterhaus *et al.*, 1988; Visser *et al.*, 1993a) which had apparently not been infected with these viruses in the last decades. Whether the crossing of species barriers is a natural phenomenon for morbilliviruses that has largely been overlooked in the past, or whether predisposing factors have rendered the species involved more susceptible to infections with 'foreign' morbilliviruses, remains to be determined. Efforts are in progress to isolate the CDV strain causing mortality among lions in the Serengeti and further sequence its H and F glycoprotein genes for more detailed comparisons.

Efficient control of morbillivirus infections can be achieved by vaccination measures. However, vaccination of wild life populations is not generally recommendable. Furthermore, use of live attenuated CDV vaccines in non-canid wildlife species which are highly susceptible to CDV infections such as the lesser panda or mustelid species like the black-footed ferret led to vaccine-induced fatal distemper. Therefore, in order to reduce the potential risk of recurrent introduction of CDV into the population of large felids and other CDV susceptible carnivores of the Serengeti we propose to vaccinate the local domestic dog population with proven safe CDV vaccines.

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Section 3.3

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

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Abstract — 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 viremia along with pronounced lymphopenia but did not show any clinical symptoms. Plasma neutralizing antibody titers 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 ten percent scattered predicted amino acid changes including up to four additional potential N-linked glycosilation 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 USA (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 *Paramyxoviridae* family, 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.*, 1993a). 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.*, 1995a). 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 USA 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 the North American zoos, the infection probably spread from cat to cat although 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 for 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 SPF domestic cats with tissue homogenate from a Chinese leopard that had died recently from a CDV infection in a zoo in the USA (Appel *et al.*, 1994) and the phylogenetic comparison of CDV isolates from felid and non-felid origin.

Materials and 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.*, 1993a), stimulated overnight in the presence of 20 μg ml^{-1} of PHA-M and maintained in Iscove's medium supplemented with 20% fetal calf serum (FCS) and 100 I.U. ml^{-1} of recombinant human interleukin 2 (rh IL-2). Feline PBMC and thymocytes or lion (*Panthera leo*) PBMC were stimulated for three days using Concanavalin A (Con A, 5 μg ml^{-1}) and expanded in RPMI 1640 containing 10% FCS and 100 I.U. ml^{-1} 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*) of the Shambala Wildlife Waystation, Angeles National Forest, California, 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 of 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). The CDV isolate A92-27/14b was obtained from a racoon (*Procyon lotor*) which had been found in close vicinity of 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 specific pathogen-free (SPF) domestic European short hair cats were inoculated intratracheally with 3 ml of 10% (w/v) tissue homogenates (lung, thymus, brain) of 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 along with the recording of rectal temperatures. PBMC were recovered for virus isolation from heparinized blood samples by lymphoprep density centrifugation as previously described (Siebelink *et al.*, 1990). Plasma was used for serological analysis. Routine hematological 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 of 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 hours in the presence of 5 μg Con A and then diluted in duplicate in five $\log_{10}$ steps from 100,000 to 1 cell per ml. 1 x 10^5 stimulated canine PBMC were added and cocultivated for seven days. Plasma samples, pharyngeal swabs and tissue homogenates of various organs were directly inoculated into canine PBMC cultures containing approx. 1 x 10^5 cells ml^{-1}. Following an adsorption period of two hours, the cells were washed twice in Iscove's medium and cultivated for seven days. After one freeze/thaw cycle a second passage of seven days was initiated for all isolation attempts.

Morbillivirus antigen was detected with the monoclonal antibody (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.*, 1993b).

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 as well as Vero and CRFK cells.

Serological assays — Plasma samples were assayed for morbillivirus neutralizing antibodies against 50 - 100 $TCID_{50}$ of the Vero cell-adapted homologous CDV isolate (A 92-27/4, 3. passage) and the Bussell strain of CDV by a microneutralization assay. ND_{50} titers were calculated by the Kaerber method (Kaerber, 1931).

Radioimmunoprecipitation assays — Vero cells infected by the respective morbilliviruses were metabolically labelled for two hours with [^{35}S]-methionine/cysteine (Amersham-Buchler, 100 μCi/25cm^2 tissue culture flask) when a viral-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 hemagglutinine (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.*, 1993b). 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.*, 1993a). All PCR reactions were limited to 25 cycles comprising of 30" at 94°C, 1' at 55°C and 1' at 72°C. A final elongation phase of 10' 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 acc. no. #D00758). Amplificates of approximately 1.9 kB comprising the complete coding region were cloned into a ßtickyT 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 17mers (H gene). Two clones obtained from a single PCR reaction were sequenced in 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 Genetics Computer Group (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 neighbor-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).

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

Results

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

Within the 36 day observation period following inoculation with tissue homogenate of 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 to 11 was observed in both these cats (Fig. 1).

Lymphopenia was accompanied by a PBMC associated CDV viremia which peaked about one week after infection and had subsided within two weeks after infection (Fig. 1). CDV was isolated in mitogen-stimulated canine PBMC: large multinucleated syncytia became apparent after four to six 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, 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 titers 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).

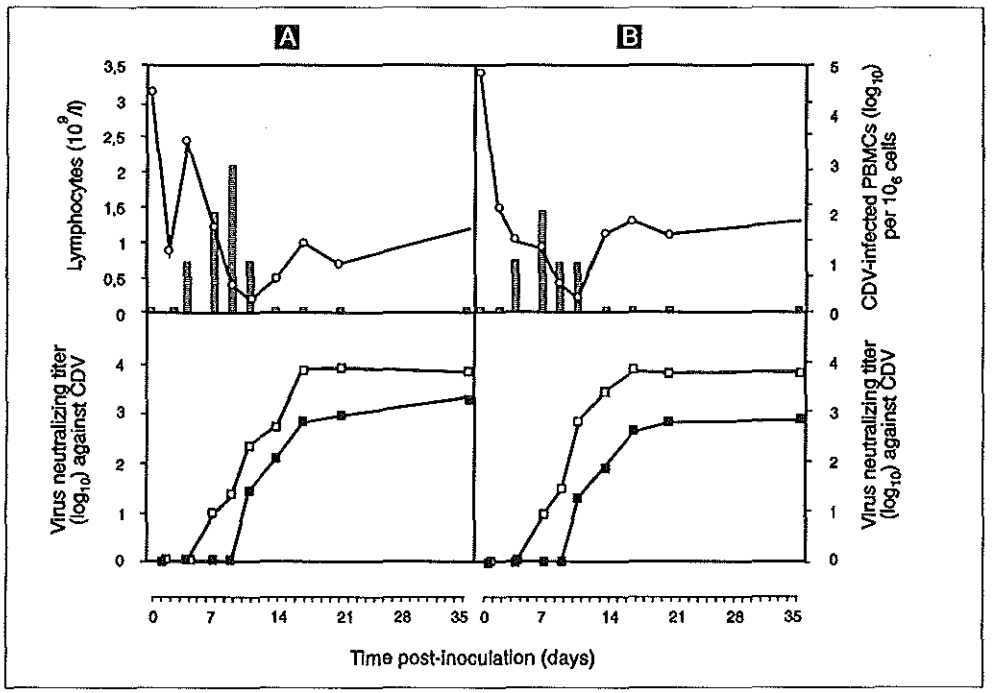


Figure 1. Results of experimental inoculation of two domestic SPF cats (A, B) with tissue homogenate from a Chinese leopard that had died from a CDV infection. Upper panel: Peripheral blood lymphocyte counts (o—o) and numbers of CDV infected PBMC per 10^6 PBMC are shown (grey columns). Lower panel: Kinetics of neutralizing plasma antibody titers (log_{10} ND_{50}) against the Chinese leopard CDV isolate A92-27/4 (□—□) and the CDV vaccine strain Bussell (■—■).

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 to 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 that of previously examined CDV strains which migrate at about 72-75 kDa (Örvell, 1980; Rima *et al.*, 1991; Shapshak *et al.*, 1982). Also, a difference between the molecular weights of the H protein of the Bussell strain (72 kDa) and A92-27/4 (78/82 kDa) was noticed (not shown).

```
                1                                                   100
Onst            AAGAGGTTAAGGGAATCGAAGATGCTGACAGTCTCGTGGTACCTGCAGGCACTGTCGGTAATCGAGGATTCGAGAGAGGAGAAGGAAGCCTTGATGATAG
Bussell         ....................................................................................................
A92-27/4        ...............................................G.....A.....G.....................................
A92-27/14b      ...............................................G.....A.....G.....................................
A92-9a          ...............................................G.....A.....G.....................................
A92-6           ...............................................G.....A.....A.....................................
5804/90         ...............................................G.....A.....................................
1493/89         ..............................C................G.....A.....................................
PDV-2           ...............................................G.C...A.....................................
94-28/PLE       ..................C.............................A..G.....A.....................................A.....

                101                                                 200
Onst            CACTGAGGATTCTGGCGAAGATTATTCCGAAGGAAATGCTTCATCTAACTGGGGATATTCTTTCGGCCTTAAACCGGACAGAGCAGCTGATGTGAGCATG
Bussell         ....................................................................................................
A92-27/4        ............................g.......................................a.....................
A92-27/14b      ............................g.......................................a.....................
A92-9a          ............................g.......................................a.....................
A92-6           ............................g.......................................a.....................
5804/90         ............................g.......................................a.....................
1493/89         ............................g.......................................a.....................
PDV-2           ....................................................................a.....................
94-28/PLE       ............................g.......................................a.....................

                201                                                 300
Onst            CTGATGGAAGAGGAATTAAGTGCTCTACTCAGGACAAGCAGAAATGTAGGGATTCAGAAAAGGGATGGGAAGACTCTGCAGTTCCCACATAATCCCGAAG
Bussell         ....................................................................................................
A92-27/4        ..............g..g........g.........................a.....................................c.....a....
A92-27/14b      ..............g..g........g.........................a.....................................c.....a....
A92-9a          ..............g..g..................A..............a.....................................c.....a....
A92-6           ..............g........g....A.....................a.....................................c.....a....
5804/90         ..............g........g....A.....................................a.....................c.....
1493/89         ..............g..c.....g..............G.....................................................c.....
PDV-2           ..............g........g.....................................G.....a.....................c.....
94-28/PLE       ..............g........g.....................................................................c.....a....

                301                                                 388
Onst            GTAAGACAAGGGATCCGGAGTGTGGATCCATTAAAAAGGGCACAGAAGAGAGGTCAGTCTCACATGGAATGGGGATAGTTGCTGGATC
Bussell         ....................................................................................
A92-27/4        ........G.....................................G...........C.....................
A92-27/14b      ........G.....................................a....G.....C.....................
A92-9a          ........G.....................................G...........C.....................
A92-6           ........G.........G.....................t....G...........C.....................
5804/90         ........G................g.....................G...........C..............A.....
1493/89         ........G.....................................G...........C.....................
PDV-2           ........G.a...................................G....G......C.....................
94-28/PLE       ........G.....................A...............G........T..C.....................
```

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 infering 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.*, 1995a) from non-felid species, a P gene sequence obtained from a PCR amplificate of CNS 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. 3a). 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 geographic origin as 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 in 3.0 % at the nucleotide level. A single large open reading frame (ORF) was identified of which the amino acid composition was deduced and aligned to those of CDV vaccine strains (Fig. 4) showing scattered predicted amino acid changes of up to 10%. While the Onderstepoort

Figure 2. Alignment of 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 acc. 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.* (1995a), respectively: 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.

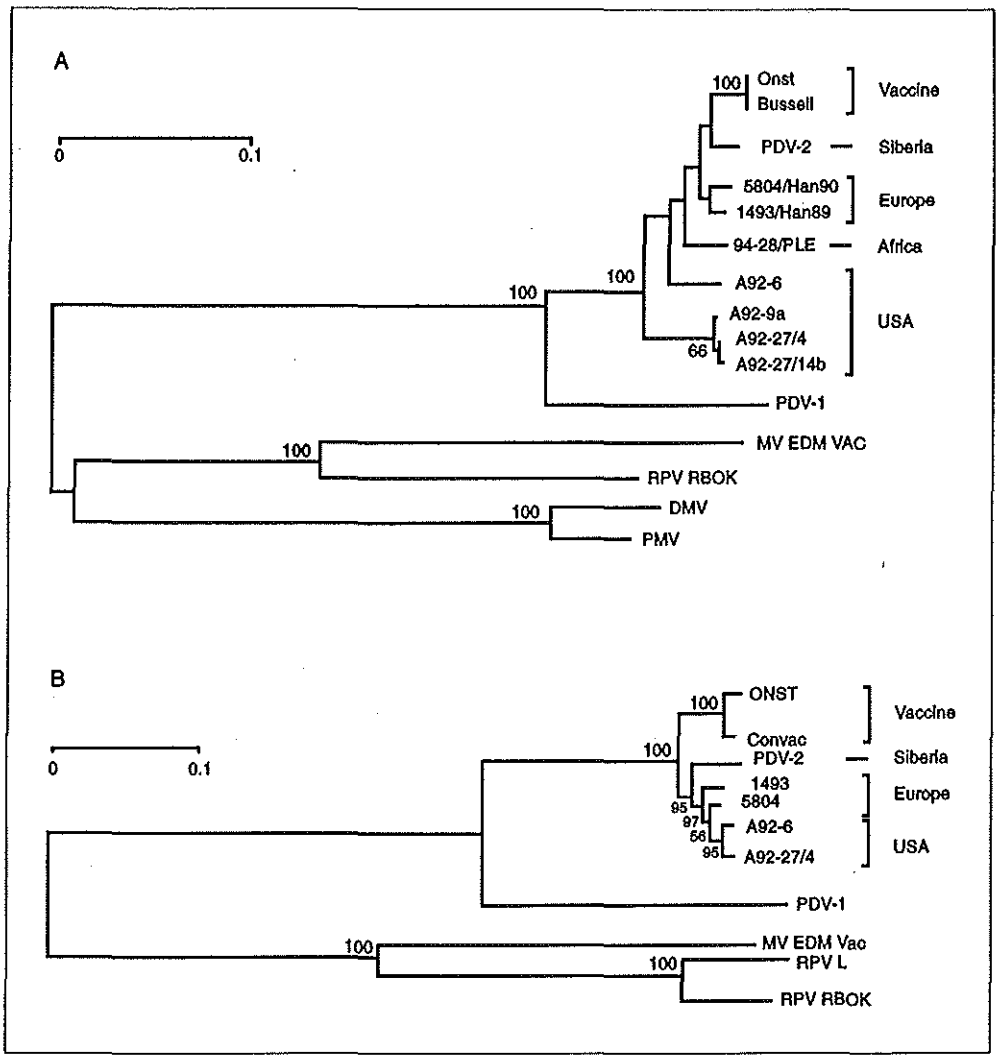


Figure 3. Phylogenetic analysis of canine distemper-like virus isolates and other morbillivirus species computed on the basis of a sequence analysis of a 388 bp P gene fragment (**A**) and the entire protein coding region of the H gene (**B**). Sequences of CDV Bussell, A92-27/4 (Chinese leopard, California 1992), A92-6 (black leopard, Illionois 1992, H gene) and A92-27/14b (raccoon, California, 1992) were generated in this study. Other sequences were extracted from GenBank database: CDV strain Onderstepoort (P: #X51869, H: #L13194), CDV strain Convac (H: #Z35493), PDV (P, H: #D10371), MV vaccine strain Edmonston (P: #M89920, H: #U03669), RPV strain RBOK

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 glycosilation pattern, however, differed considerably (Fig. 4). While four potential N-linked glycosilation sites were shared between all CDV isolates, three (Convac and A92-6), respectively four (A92-27/4) additional sites, all located in the extracytoplasmic domain, proved to be present in the other sequences.

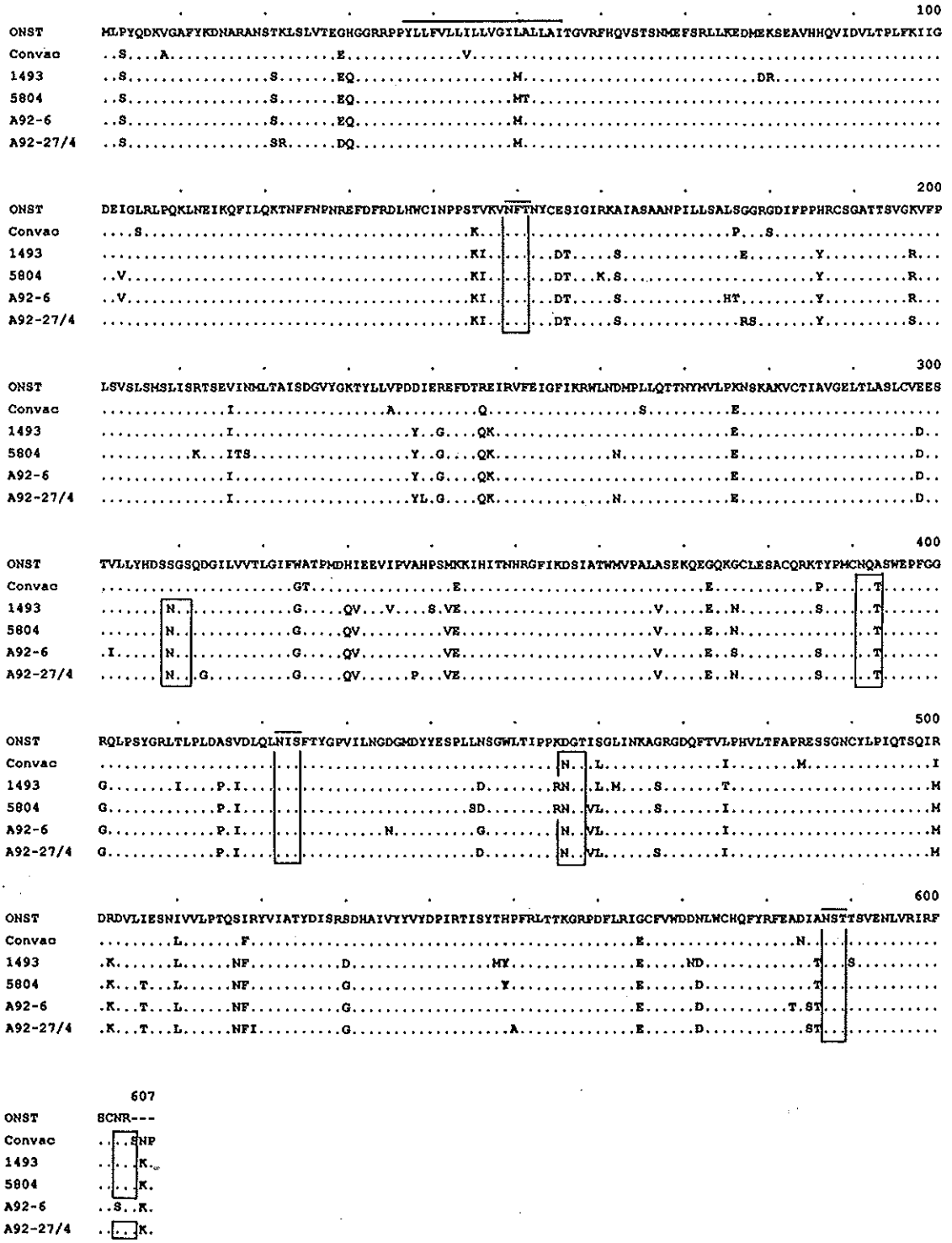
Phylogenetic analysis of the entire protein-encoding region of the H gene (Fig. 3b) confirmed the clustering of the large cat isolates as CDV-like. Bootstrap *P* values indicate that they are significantly separated from CDV vaccine strains and also from current European and Asian CDV wild-type isolates.

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 this inoculation as it has been shown that adaptation of morbilliviruses to replication in cell culture systems may affect its 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 that e.g. infection with the usually highly virulent Snyder-Hill

(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.*, 1995a, 1995b): 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 neighbor-joining method (Saitou & Nei, 1987). Alignment gaps were ignored in pairwise distance 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 $\geq$ 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).


100
ONST MLPYQDKVGAFYKDNARANSTKLSLVTEGHGGRRPPYLLFVLLILLVGILALLAITGVRFHQVSTSNMEFSRLLKEDMEKSEAVHHQVIDVLTPLFKIIG
Convac
1493
5804
A92-6
A92-27/4
200
ONST DEIGLRLPQKLNEIKQFILQKTNFFNPNREFDFRDLHWCINPPSTVKVNFTNYCESIGIRKAIASAANPILLSALSGGRGDIFPPHRCSGATTSVGKVFP
Convac
1493
5804
A92-6
A92-27/4
300
ONST LSVSLSHSLISRTSEVINMLTAISDGVYGKTYLLVPDDIEREFDTREIRVFEIGFIKRWLNDMPLLQTTNYMVLPKNSKAKVCTIAVGELTLASLCVEES
Convac
1493
5804
A92-6
A92-27/4
400
ONST TVLLYHDSSGSQDGILVVTLGIFWATPMDHIEEVIPVAHPSMKKIHITNHRGFIKDSIATWMVPALASEKQEGQKGCLESACQRKTYPHCNQASWEPFGG
Convac
1493
5804
A92-6
A92-27/4
500
ONST RQLPSYGRLTLPLDASVDLQLNISFTYGPVILNGDGMDYYESPLLNSGWLTIPPKDQTISGLINKAGRGDQFTVLPHVLTFAPRESSGNCYLPIQTSQIR
Convac
1493
5804
A92-6
A92-27/4
600
ONST DRDVLIESNIVVLPTQSIRYVIATYDISRSDHAIVYYVYDPIRTISYTHPFRLTTKGRPDFLRIGCFVWDDNLWCHQFYRFEADIANSTTSVENLVRIRF
Convac
1493
5804
A92-6
A92-27/4
607
ONST SCNR---
Convac
1493
5804
A92-6
A92-27/4

strain of CDV only results 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 can not 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 (Osterhaus & 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 hardly 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 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 ten percent scattered differences, which is within the range reported for other morbillivirus species like MV and RPV. The number of potential N-linked glycosilation sites in the H protein of the large cat isolates resembles the situation of recent wild-type CDV isolates (5804, 1493) of non-felid origin (Mamaev *et al.*, 1995b), 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 glycosilation 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. Comparable differences in cross neutralization efficacies were also described for recent CDV field isolates from a dog (5804)

Figure 4. Alignment of deduced amino acid sequences of the H-proteins the canine distemper virus vaccine strain Onderstepoort (#D00758), the 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-glycosilation sites are boxed, the site at position 19 (dotted box) is probably not used as it is located in the intracellular domain.

and a ferret (1493) using sera raised aginst the Rockborn vaccine strain of CDV in a kinetic neutralization assay (Harder *et al.*, 1993a). An explicit clustering of predicted amino acid changes around potential Asn-glycosilation sites, as has been described for recent MV field isolates (Rota *et al.*, 1992), however, was neither found 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 geographic rather than to host species origin was noticed. Geographic lineages have also been described for other morbillivirus species (Chamberlain *et al.*, 1993; Rota *et al.*, 1992; Taylor *et al.*, 1991). On basis of our data it can be excluded that the US large cat isolates have recently evolved from CDV vaccine strains. Instead they rather seem to be representatives of CDV strains presently circulating in North America, which was 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 obtained so far do not indicate the existence of a CDV lineage adapted to feline species but rather 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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Chapter 4

Discussion

Section 4.1

Herpesviruses of pinnipeds: Clinical and virological implications

Timm C. Harder and Albert D.M.E. Osterhaus

Abstract — Harbour seals (*Phoca vitulina*) are host to an α- and a γ– herpesvirus, designated phocid herpesvirus type-1 and type-2 (PhHV-1, -2). Serosurveys in various pinniped species revealed a global prevalence of PhHV-1 and, in part, also of PhHV-2. PhHV-1 has been shown to exhibit a significant pathogenic potential, particularly in hospitalized seal populations. Disease varies considerably from mild upper respiratory disorders to fatal generalized exacerbations and is inversely correlated to the age and the immunocompetence of the affected individual. Antigenic and molecular characterizations showed that the herpesviruses of terrestrial carnivores, felid and canid herpesvirus, are closely related to PhHV-1. Recent developments in the diagnosis of PhHV-1 infections and attempts toward a subunit vaccine protecting seals from PhHV-1-associated disease are discussed. As yet, no disease conditions have been ascribed to PhHV-2 infection. Previous antigenic and molecular studies and further nucleotide sequence analysis presented here, suggest a clustering of PhHV-2 together with its seemingly closest relative, the equine herpesvirus-2, as a third genus in the γ–*Herpesvirinae* subfamily.

Introduction

Devastating epizootics among seals in northwestern European waters (1988) and among dolphins of the Mediterraenean Sea (1990/91), caused by previously unknown viruses of the *Morbillivirus* genus, resulted in an upsurge of general public and scientific interest in virus infections of aquatic mammals (Heide-Jørgensen *et al.*, 1992; Osterhaus *et al.*, 1995). A considerable expansion in knowledge of marine mammal viruses has occured since. To date, at least thirteen virus species out of ten virus families, including, e.g., adeno-, pox-, morbilli- and herpesviruses, have been shown to infect pinnipeds and/or cetaceans (reviewed by Visser *et al.*, 1991). While morbilliviruses require a considerable critical population size of up to 400.000 individuals to maintain an endemis status, herpesviruses, in contrast, perpetuate even in small populations. Perpetuation is related to persisting latent infections induced by herpesviruses in susceptible hosts; infectious virus can be periodically reactivated from latency and is often asymptomatically shed. Diseases provoked upon primary herpesvirus infection vary from mild superficial mucutaneous lesions, respiratory and lymphoproliferative disorders to fatal generalized infections. Severity of disease is modulated by the age and the immunocompetence of the infected individual.

Pinnipeds are host of at least two distinct herpesvirus species. The α-herpesvirus of seals, referred to as phocid herpesvirus-1 (PhHV-1), is recognized since 1985 as an important pathogen of free-ranging and hospitalized harbour seals (*Phoca vitulina*) (Osterhaus *et al.*, 1985; Frey *et al.*, 1989; Stenvers *et al.*, 1992a). We recently showed that PhHV-1 is by far the most frequently encountered viral pathogen in hospitalized newborn and juvenile harbour seals (Harder *et al.*, 1997a), where enzootic PhHV-1-related disease outbreaks can account for mortality rates of up to 50% (Osterhaus *et al.*, 1985).

A second seal herpesvirus type, biologically and antigenically markedly distinct from PhHV-1, has been isolated from a captive California Sea lion (*Zalophus californianus*) and from adult

harbour seals of European (North Sea) and United States (North Atlantic) waters and has been designated PhHV-2 (Kennedy-Stoskopf *et al.*, 1986; Lebich *et al.*, 1994; Harder *et al.*, 1996a). While previously published nucleotide sequence data of PhhV-2 suggested a classification as a member of the γ-*Herpesvirinae*, the limited data did not allow an unambiguous clustering into one of the γ–herpesvirus genera (Harder *et al.*, 1996a).

Here, we review data published on the clinical impacts and the molecular properties of herpesviruses of pinnipeds. In addition, novel nucleotide sequence data of PhHV-2 are presented that enable a more precise taxonomic and phylogenetic classification.

PhHV-1 — a pathogenic alpha-herpesvirus of seals

PhHV-1 has been first described in 1984 as an agent causing an epizootic disease that was associated with high mortality rates among newborn and juvenile harbour seals housed for rehabilitation in a Dutch seal sanctuary (Osterhaus *et al.*, 1985). Up to now, PhHV-1 continued to be the most frequent viral pathogen isolated from hospitalized seals (Harder *et al.*, 1997a). Development of disease following primary PhHV-1 infection depends largely on the age and the immunocompetence: Clinically healthy juvenile and adult seals will develop mild upper respiratory disease which is usually self-limiting. Inapperent infections probably occur frequently as has been shown by a transmission experiment using low passage cell culture-grown PhHV-1, which provoked only mild temperature rises in juvenile seals (Horvat *et al.*, 1989). Whether also fertility problems and ocular disease, disorders commonly described for various α–herpesvirus infections, are also associated with PhHV-1 infection remains to be elucidated. Severe and frequently fatal PhHV-1 disease is seen in immunoincompetent (neonates) and otherwise immunocompromised seals which are prone to fulminant generalized infections characterized by focal necrotic hepatitis and nephritis, gastroenteritis, bronchopneumonia and encephalitis (Borst *et al.*, 1986).

The majority of PhHV-1 isolates has been obtained from diseased adult seals during the 1988 European seal mass mortality (Frey *et al.*, 1989; Have *et al.*, 1991). Fatal generalized PhHV-1 infection in these seals probably arose endogeneously from a reactivated latent PhHV-1 status as a sequel of the concurrent phocid distemper morbillivirus (PDV) infection in these animals, being at the basis of a severe immunosuppression (Osterhaus & Vedder, 1988; Osterhaus *et al.*, 1988; Zhang *et al.*, 1989; Haas *et al.*, 1991). Hardly any data are available on the incidence of PhHV-1 associated diseases in free-ranging pinniped populations, independent from morbillivirus epizootics. It should be noted, however, that PhHV-1 has been repeatedly isolated since 1990 from carcasses of newborn and adult harbour seals and grey seals (*Halichoerus grypus*) which were found ashore the Baltic and the North Seas as well as along US Atlantic coasts (pers. comm. by M. Appel and K. Kulonen; Harder, unpublished; Harder *et al.*, 1991a, 1996a).

Diagnosis of PhHV-1 infection is accomplished by demonstration of at least fourfold rises of virus neutralizing antibody titers, by virus isolation from swabs and tissue suspensions

or by PCR (Osterhaus *et al.*, 1985; Frey *et al.*, 1989; Harder *et al.*, 1997a). The recently developed PCR assay, targeting a fragment of the gD gene equivalent of PhHV-1, has been shown to be surperior to virus isolation with respect to sensitivity, particularly when bacteriologically contaminated or degraded specimens are to be examined (Harder *et al.*, 1997a). A comprehensive panel of monoclonal antibodies (mAbs) is available for the rapid identification and antigenic characterization of putative herpesvirus isolates from pinnipeds (Lebich *et al.*, 1994).

In vitro, the host cell spectrum comprises primary seal fibroblast and epithelial cultures (lung/kidney) and several feline cell lines (Crandell-Rees feline kidney, *Felis catus* whole fetus cells) as well as primary lung or kidney cultures of cats. In cell culture, PhHV-1 exhibits a comparatively short replication cycle and significant amounts of cell-free infectivity are released. Maximum infectivity titers peak at about $10^{5.0}$ $TCID_{50}$ (Lebich *et al.*, 1994). Round cell formation constitutes the predominant cytopathic effect induced by PhHV-1.

Antigenically, PhHV-1 has been shown to be closely related to canid herpesvirus (CHV), and to a lesser extend, also to felid herpesvirus (FHV) (Osterhaus *et al.*, 1985; Harder *et al.*, 1991a; Stenvers et al., 1992b). Immunogenic proteins expressed in infected cells by all authentic α–herpesviruses of carnivores (FHV, CHV and PhHV-1) exhibit comparable patterns and molecular weights (Harder *et al.*, 1997b). Differentiation is possible by cross neutralization assays and by typing with mAbs (Lebich *et al.*, 1994).

Further evidence for a classification of PhHV-1 as a separate species in the *Varicellovirus* genus of the α–*Herpesvirinae* is based on nucleotide sequence and subsequent phylogenetic analysis of the gene encoding the glycoprotein B (gB) which is conserved among all herpesvirus species (Harder & Osterhaus, 1997). On basis of this analysis, CHV represents the closest relative of PhHV-1 although *in vitro*, canine cells, in contrast to cultures of feline origin, are refractory to PhHV-1 and *vice versa*. In addition, gB has been demonstrated a major immunogenic protein of PhHV-1 and carries neutralization-relevant epitopes (Lebich et al., 1994; Harder & Osterhaus, 1997; Harder et al., 1997b). PhHV-1 gB has been successfully expressed as an immunogenically and antigenically authentic molecule in insect cells (Harder & Osterhaus, 1997).

According to several serosurveys, infections with PhHV-1 or other closely related α–herpesviruses occur globally in various pinniped species including those of Arctic and Antarctic habitates (Vedder *et al.*, 1987; Frey *et al.*, 1989; Harder *et al.*, 1991a; Stenvers *et al.*, 1992b; Stuen *et al.*, 1994; Zarnke *et al.*, 1997). Seroprevalences, however, ranged widely in different areas and species.

Little is known to date about the epidemiology of PhHV-1. In analogy to other α–herpesviruses, however, PhHV-1-seropositive seals are considered life-long carriers of latent virus which can be periodically reactivated and may be shed asymptomatically. Spread of infection is assumed to occur predominantly horizontically via aerosols and direct contact. Considering the close antigenic relationship between PhHV-1, CHV and FHV, a susceptibilty of terrestrial carnivores to PhHV-1 cannot be ruled out at present, although recent attempts to transmit PhHV-1 experimentally to FHV-seronegative domestic cats have failed (Osterhaus & Soethout, unpublished).

Gamma-herpesvirus infections of pinnipeds

Herpesviruses that are antigenically and biologically distinct from PhHV-1 have been isolated since 1986 from four harbour seals (ranging in US North Atlantic waters and in the North Sea, respectively) and from a hospitalized Californian sea lion (*Zalophus californianus*) which suffered from a terminal, respiratory disease (Kennedy-Stospkopf *et al.*, 1986; Lebich *et al.*, 1994; Harder *et al.*, 1996a). Isolation apparently required the direct cultivation of tissues or peripheral mononuclear blood cells (PBMCs) and subsequent cocultivation with primary seal fibroblast cultures. Adaptation to growth on feline cell lines and - at least for two isolates - also to a simian cell line (Vero) has been achieved (Harder *et al.*, 1996a). In contrast to PhHV-1, these isolates frequently induced giant cell formation, exhibited delayed replication kinetics and their infectivity remained largely cell-associated (Lebich *et al.*, 1994). Antigenic characterization by cross neutralization, typing with mAbs and analysis of viral proteins expressed in infected cells further confirmed their diversity from PhHV-1, CHV and FHV (Lebich *et al.*, 1994; Harder *et al.*, 1996a, 1997b). Since all five isolates examined so far reacted homogeneously with a panel of mAbs raised against the European isolate '7848', and all, except one, are of harbour seal origin, they are referred to as phocid herpesvirus type-2 (PhHV-2).

Previous analysis of nucleotide sequence data from a partial *Eco*RI library of the PhHV-2 genome (isolate 7848) revealed greatest homology to members of the γ–*Herpesvirinae* (Harder *et al.*, 1996a). In order to confirm and specify the classification of PhHV-2 as a γ–herpesvirus, here we report on the extension of nucleotide sequence analysis from this library:

Further *Eco*RI clones were chosen arbitrarily and various subclones, not exceeding 600 bp in length, were constructed in pBluescript. M13 forward and reverse primers were used in dye-dideoxy cycle sequencing reactions (Perkin-Elmer). Semiautomated sequencing was performed on a A373 sequencer (Applied Biosystems). Assembly and analysis of sequence data were carried out with the GCG software package version 8.0 (Devereux *et al.*, 1984). Sequences were generated from fragments *Eco*RI 78e22, 78e11a and 78e11b which received GenBank accession nos. Z83323, Z83322 and Z83324. Open reading frames (ORFs) detected in these fragments ('Frame', GCG) were translated and the deduced amino acid (aa) sequences used in a 'FastA' search of the SwissProt and PIR databases. Significant homology to proteins encoded by genes 24 (minor capsid protein: 78e11a), 25 (major capsid protein: 78e11b) and 56 (part of helicase/primase complex: 78e22), respectively, of γ-herpesviruses were revealed (Albrecht *et al.*, 1992; Telford *et al.*, 1995). Pairwise aligments with homologous sequences of species of other herpesvirus subfamilies were compiled ('Gap', GCG). Identity and similarity scores between the aligned sequences are summarized in Table 1 and confirm that PhHV-2 displays greatest homology to members of the γ–*Herpesvirinae*.

Further proof to classify PhHV-2 as a γ–herpesvirus was obtained by analysing the CpG dinucleotide frequencies of these sequences. As an exception among viruses with a large genome, γ–herpesviruses, capable of establishing latency in dividing (lymphoid) cells, have been reported to be suppressed in the dinucleotide CpG which is compensated for by a

Table 1. Comparison of ORFs deduced from PhHV-2 specific sequences.

PhHV-2 fragment	Virus species	Gene equivalent[1]	Comparisons [2]		
			%Homology	%Identity	Gaps
78e22[3]	Equine herpesvirus-2 (EHV-2)	ORF 56	66	52	2
(237 aa)	Herpesvirus saimiri-2 (HVS-2)	ORF 56	65	52	1
	Epstein-Barr virus (EBV)	BSLF1	45	29	2
78e11a[4]	Equine herpesvirus-2	ORF 24	47	35	2
(269 aa)	Herpesvirus saimiri-2	ORF 24	48	36	4
	Human herpesvirus-8 (HHV-8)	ORF24H	49	36	4
78e11b	Equine herpesvirus-2	ORF 25	80	67	1
(397 aa)	Herpesvirus saimiri-2	ORF 25	74	61	1
	Human herpesvirus-8	ORF 25H	74	61	1
	Epstein-Barr virus	BcLF1	66	54	1
	Human cytomegalovirus (HCMV)	UI86	47	31	12
	Human herpesvirus-6 (HHV-6)	U57	45	31	9
	Herpes simplex virus-1 (HSV-1)	UI19	44	29	11
	Equine herpesvirus-1 (EHV-1)	ORF 42	43	27	9
	Varicella zoster virus (VZV)	ORF 40	42	26	10

[1] Sequences for comparisons were extracted from the GenBank databases: EHV-2 #U20824; HVS-2 #Q01006, #P14346; EBV #X00784; HHV-8 #U40377; HHV-6A #X83413; HCMV #X17403; EHV-1 #M86664; VZV #P09270.

[2] Deduced amino acid sequences were compared by using the 'Gap' program of the GCG software package, setting gap weight and gap extension values to 3.0 and 0.1, respectively. The calculation of similarity values is based on the Dayhoff matrices (Dayhoff *et al.*, 1983).

[3] Homology values for equivalent proteins of HCMV (UI70), HHV-6 (U43), EHV-1 (ORF 7), VZV (ORF 6), HSV-1 (UI52) was estimated less than 30% in the corresponding parts.

[4] No ascertained translation for corresponding part of EBV (BcRF1) available; no homologues in α–herpesviruses.

relative excess of TpG and CpA, respectively, relative to their mononucleotide frequencies (Honess *et al.*, 1989). It has been postulated that CpG suppression in these viral genomes may be driven by high *de novo* methylating activities in the nucleus of dividing cells, leading to methylation of the cytosine residues in CpG dinucleotides. 5-methylcytosine is then desaminated to give thymine (Honess *et al.*, 1989). Contradictory to the methylation-desamination-mutation hypothesis, however, the genomes of the T-lymphotropic Marek's disease herpesvirus and the human herpesvirus-6 (an α– and a β–herpesvirus, respectively) are not suppressed in CpG frequencies despite the fact that they, biologically, share a number of features with γ–herpesviruses. Herpesviruses which establish latency in postmitogenically fixed cells such as neurons, in general, are not forced into CpG suppression (e.g. HSV-1

Table 2. CpG dinucleotide frequencies in PhHV-2 gene fragments.

Fragment (GenBank acc. no.)	Dinucleotide frequencies [1]		
	CpG	TpG	CpA
78e22 (U54678)	0.27 [2]	1.37	1.25
78e11a (U45682)	0.35	1.28	1.17
78e11b (U45679)	0.32	1.63	1.27
UI52-H[3] (X34567)	0.15	1.35	1.17
ORF23-H[3] (X34521)	0.22	1.61	1.33

[1] P-values, as a measure of the odds-ratios of frequencies were calculated according to Karlin *et al.*, 1994b).

[2] For values ≤ 0.78 the relative dinucleotide abundance is regarded to be low, whilst p-values ≥ 1.25 indicate a relative excess of the particular dinucleotide (Burge *et al.*, 1992).

[3] Previously obtained from the PhHV-2 (isolate 7848) *Eco*RI library (Harder *et al.*, 1996a).

or VZV). When PhHV-2-specific sequences were analysed for CpG ratios according to the method described by Karlin *et al.* (1994b), a significant CpG suppression and a relative excess of TpG and CpA was evident (Tab. 2), similar to other γ–herpesviruses.

Possible evolutionary relationships of PhHV-2 were analysed using the deduced aa sequences of the major capsid protein (MCP) fragment which is known to be fairly conserved among all species of herpesviruses (McGeoch *et al.*, 1995). A total of 281 out of 397 sites of the PhHV-2 MCP fragment could be reliably aligned when compared with herpesvirus species derived from all subfamilies. In this analysis, the herpesvirus subfamilies and the γ–herpesvirus genera were correctly assigned (Fig. 1a). In order to refine the clustering patterns and to obtain greater statistical robustness, γ-herpesvirus sequences were aligned separately, using 396 out of 397 sites of the PhHV-2 MCP fragment.

According to this analysis, PhHV-2 shows particular affinity to EHV-2 (Fig. 1b) which has been provisionally classified a γ_3-herpesvirus (Telford *et al.*, 1993; 1995). Unfortunately, there is still insufficient sequence data of this region from other γ–herpesviruses of non-primate origin such as, e.g., bovid herpesvirus-4 (which has also been sporadically isolated from cats) and the alcephaline herpesviruses to allow for comparisons on a broader base.

Little is known concerning the seroprevalence of PhHV-2. Neutralizing antibodies specific for PhHV-2 have been detected in a small cohort of harbour seals of the North Sea and, at moderate prevalence, in several pinniped species ranging in northern Pacific waters, but not in Weddell seals (*Leptonychaetes weddellii*) of Antarctica (Harder *et al.*, 1991a; Lebich *et al.*, 1994; Zarnke *et al.*, 1997). Seroprevalences of PhHV-1 and -2 infections in a given pinniped species, however, differed considerably. This suggests distinct epizootiological patterns, possibly due to different transmission modes. While a primary pathogenic role of PhHV-2 in seal populations seems highly unlikely, the clinical significance of PhHV-2 infections in individual seals remains to be elucidated. In light of the fact that PhHV-2 crosses species borders *in vitro*, the pathogenic potential of PhHV-2 for terrestrial carnivores needs to be assessed.

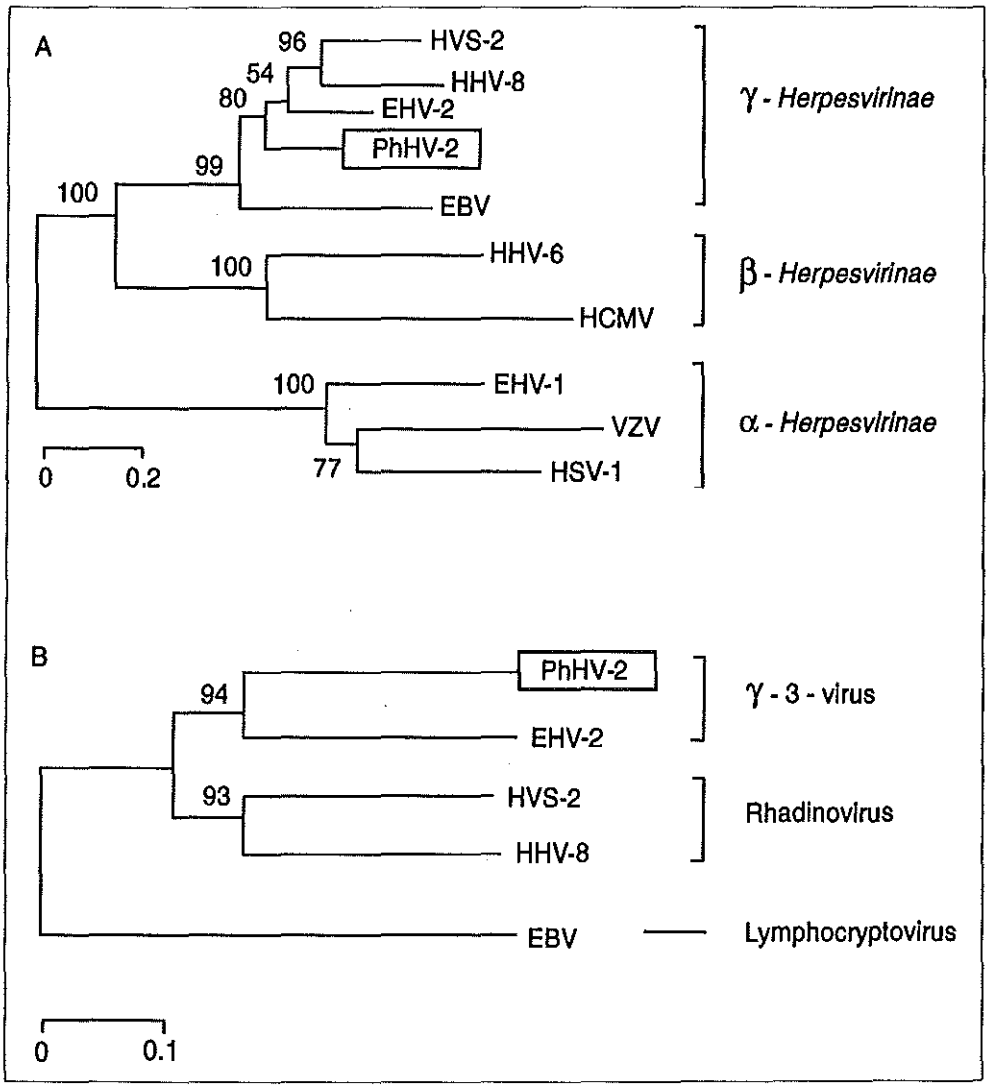

Figure 1. Analysis of phylogenetic relationships of PhHV-2 using deduced amino acid sequences of a fragment of the major capsid protein homologue (78e22, Tab. 1). Multiple alignments of herpesvirus sequences homologous to the PhHV-2 specific fragment were generated using 'Pileup' (GCG). Alignment gaps were completely omitted for computation of genetic distances which was finally based on 281 sites (Fig. 1a) and on 396 sites (Fig. 1b), respectively. Distances were calculated assuming a γ-distribution ($\gamma = 2$). Trees were constructed by the neighbor-joining principle. Bootstrap p values after 500 replications are indicated at nodes. Values ≥ 95 indicate significant separation of clusters. Phylogenetic inferences were carried out using the 'MEGA' PC software (Kumar *et al.*, 1994.

Conclusions

α–herpesviruses, identical or closely related to PhHV-1, appear to be globally prevalent in all pinniped species examined so far. While no reliable data on the clinical impact in free-ranging seal populations independent from morbillivirus epizootics exist to date, PhHV-1 continues to cause significant morbidity and mortality among hospitalized newborn seals. Several seal species such as the Hawaiian and Mediterraenean monk seals (*Monachus schauinslandii* and *Monachus monachus*) are highly endangered and various rehabilitation or breeding programmes have been launched. In order to improve the prospects of such initiatives, prevention of PhHV-1-induced disease by vaccination may be desirable. Non-replicating vaccines are generally given preference for use in wildlife species because control of modified-live vaccine viruses in the field is not feasible. Favourable experiences with α–herpesvirus subunit vaccines that consisted of the glycoproteins gB and/or gD have been made for a number of herpesviruses (Burke, 1993; Inglis, 1995): Protective immunity, related to neutralizing antibodies and cytotoxic T cells, has been induced against, e.g., bovid herpesvirus-1 infection in calves (Van Drunen Littel-van den Hurk *et al.*, 1993) and against HSV-1 infection in the mouse model (Landolfi *et al.*, 1993; Ghiasi *et al.*, 1994). Phase III trials in humans using an ISCOM-enhanced vaccine preparation of HSV-2 gD and gB are about to commence (Morein, pers. comm.). With respect to the successful expression of PhHV-1 gB in insect cells, preconditions for a subunit vaccine for use in seals are reasonable. However, in analogy to other α–herpesviruses, further components, in particular a gD equivalent, need to be included. Work on cloning and sequencing the gD gene of PhHV-1 is currently under way (Harder *et al.*, unpublished).

Since 1986, a second herpesvirus species, designated PhHV-2, has been isolated independently in at least three different laboratories. PhHV-2 has now been genetically characterized as a γ–herpesvirus with particular affinity to EHV-2. Preliminary phylogenetic analysis suggested their clustering as a third genus of the γ–*Herpesvirinae*.

Acknowlegdements — Work on seal herpesviruses in our lab was funded by grants from the the Seal Rehabilitation and Research Center, Pieterburen, The Netherlands, and by the European Community (Human Capital and Mobility Project ERBCHBGCT920106).

Section 4.2

Morbilliviruses and morbillivirus diseases of marine mammals

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Abstract — In recent years, serious disease outbreaks among seals and dolphins were attributed to infection with established or newly recognized morbilliviruses. The first identification of a morbillivirus as causative agent of a mass mortality among marine mammals was in 1988, when the previously unrecognized phocine distemper virus (PDV) caused the death of 20.000 harbour seals (*Phoca vitulina*) in northwestern Europe. A similar epizootic among Baikal seals (*Phoca sibirica*) Siberia in 1987 was later attributed to infection with canine distemper virus (CDV). A morbillivirus isolated from stranded harbour porpoises (*Phocoena phocoena*) between 1988 and 1990 proved to be yet another new member of the genus Morbillivirus, distinct from PDV and CDV and more closely related to rinderpest virus and peste-des-petits-ruminants virus: porpoise morbillivirus. A similar was the primary cause of a mass mortality among striped dolphins (*Stenella coeruleoalba*) in the Mediterranean from 1990 - 92: dolphin morbillivirus. In th present review, the current knowledge of the genetic and antigenic relationships of these viruses is presented, and the origin and epizootiological aspects of the newly discovered morbilliviruses are discussed. In addition, the possible contributory role of environmental contaminant-related immunosuppression in the severity and extent of the different disease outbreaks is discussed.

Morbillivirus infections in pinnipeds

In 1988, an apparantly contagious disease spread among harbour seals and grey seals (*Halichoerus grypus*) in northwestern Europe. Disease symptoms included respiratory, gastrointestinal and neurological disorders, and were often complicated by secondary parasitical, bacterial or viral infections. Among harbour seals, the disease was accompanied with mortality levels of more than 60% in some areas, while in grey seals mortality was relatively low. The first indication of the involvement of a morbillivirus in the outbreak came from serological studies on paired serum samples, in which CDV neutralizing antibodies were found in sera of affected animals (Osterhaus & Vedder, 1988). On the basis of these serological data, in combination with the clinical signs and pathological lesions found in infected seals which appeared to be quite similar to those observed in dogs with canine distemper, it was concluded that 'a morbillivirus closely related if not identical to CDV' was the primary cause of the outbreak (Osterhaus *et al.*, 1988). Virus isolation and subsequent characterization demonstrated that the virus was not identical to CDV, but should be considered as a new member of the genus *Morbillivirus*, now referred to as phocine distemper virus (Cosby *et al.*, 1988; Mahy *et al.*, 1988; Visser *et al.*, 1990). Finally, Koch's postulates were fulfilled when seals proved to be protected from fatal challenge infection by vaccination with an inactivated CDV vaccine, and the virus could be re-isolated from the sham vaccinated animals in this experiment (Osterhaus *et al.*, 1989a; Visser *et al.*, 1989).

After the identification of PDV as causative agent of the northwestern European epizootic, a similar outbreak among Baikal seals in Siberia (Lake Baikal), which started in 1987, could also be attributed to infection with a morbillivirus (Grachev *et al.*, 1989; Osterhaus *et al.*, 1989b). Initially, it was suspected that an epizootiological link between this virus, which was tentatively called PDV-2, and the northwestern European virus, which we will refer to here as PDV-1. PDV-2, like PDV-1, could be isolated from organ materials, and caused mild

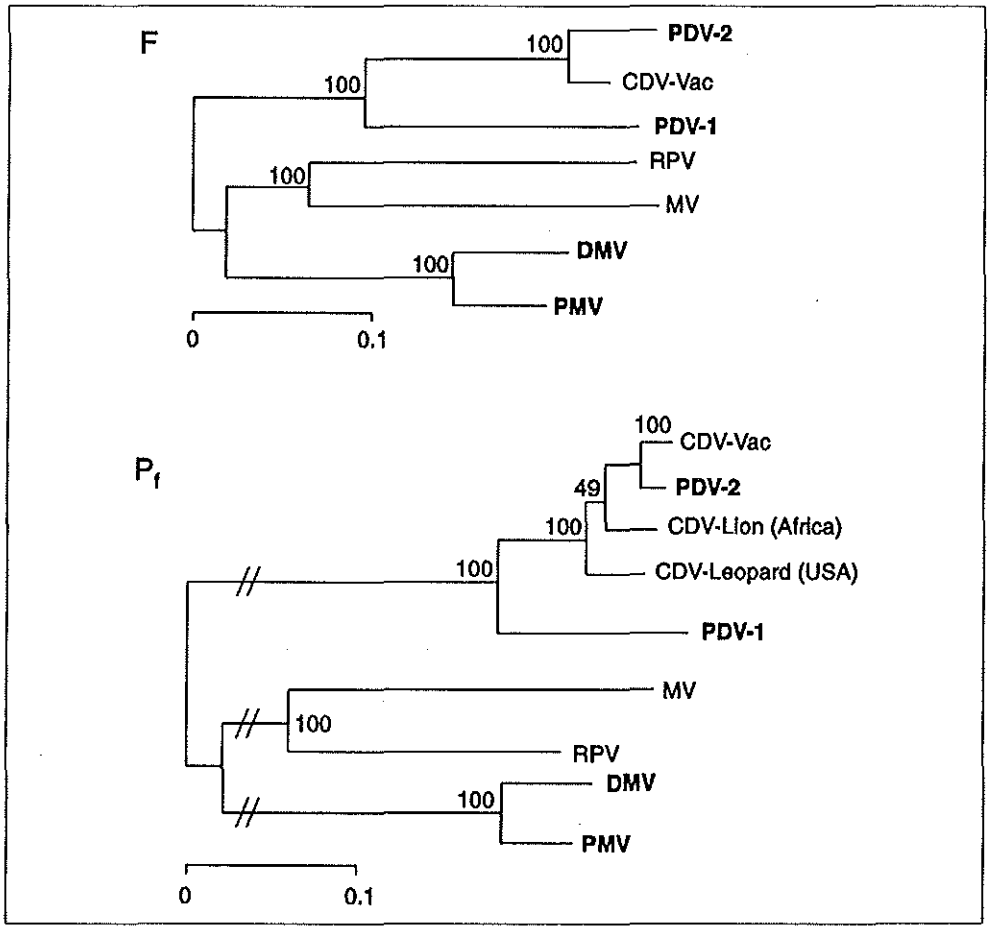
clinical symptoms in SPF dogs (Visser *et al.*, 1990). However, comparison of PDV-1, PDV-2 and CDV with respect to biological, morphological, protein chemical and antigenic properties confirmed that PDV-1 was a morbillivirus different from CDV, and revealed only minor differences between PDV-2 and CDV (Visser *et al.*, 1990). Nucleotide sequence homologies between nucleoprotein (NP), phosphoprotein (P), matrix (M), hemagglutinin (H) and fusi (F) protein genes of PDV-1 and CDV ranged from 65 to 85% (Blixenkrone.Møller *et al.* 1992; Haas *et al.*, 1991; Kövamees *et al.*, 1991; Sharma *et al.*, 1992; Visser *et al.*, 1993a). Comparison of the PDV-2 F gene with other morbillivirus F genes for which sequence data are available, revealed a 91% similarity to the complete CDV F gene, and a 97% similarity when only the coding regions of the F genes were compared. This is a level of sequence variation similar to that observed when geographically different isolates of e.g. rinderpest virus (RPV) are compared (Visser *et al.*, 1993a)

Morbillivirus infections in cetaceans

When in 1988 porpoises that had stranded along the Irish coast were shown to be morbillivirus antigen-positive (Kennedy *et al.*, 1988), and the virus involved was subsequently isolated from animals stranded along both the Irish and Dutch coasts (McCullough *et al.*, 1991; Visser *et al.*, 1993b), it was assumed that the virus isolated would be identical to PDV-1. In 1990 we also found evidence for a morbillivirus infection in a white beaked dolphin (*Lagenorhyncus albirostris*) that had stranded on the Dutch coast, by detecting morbillivirus antigen in the lungs of this animal (unpublished observation). In that same year, large numbers of striped dolphins started to wash ashore along the coasts of the western part of the Mediterranean Sea (Domingo *et al.*, 1990; Van Bressem *et al.*, 1991). The epizootic progressed eastward to reach the Turkish coast in 1992. Virus isolates were compared with PDV-1 and CDV, and with the above mentioned porpoise isolates. It was shown that the dolphin and porpoise viruses were quite similar, but both were distinct from PDV-1 and CDV. They were subsequently named dolphin morbillivirus (DMV) and porpoise morbillivirus (PMV) (Van Bressem *et al.*, 1993; Visser *et al.*, 1993b). Comparison of a sequence of the P gene encoding the RNA editing site from all presently known members of the genus confirmed that DMV and PMV

Figure 1. Unrooted phylogenetic tree of the fusion protein encoding region (F) and a fragment of the gene encoding the polymerase-associated protein (P_f) of several morbilliviruses. Genetic distances between the nucleotide sequences were calculated according to Kimura's 2-parameter method (Kimura, 1980), and used to construct phylogenetic trees employing the neighbour-joining principle (Saitou & Nei, 1987). Branch lengths are drawn in proportion to genetic distances as indicated by bars. The robustne of the tree was tested by bootstrap analysis (Felsenstein, 1985) using 2000 replications (Hedges, 1992). Numbers at nodes represent bootstrap percentages. Values greater than 95% indicate a statistically significant separation of the respective cluster from of the tree. All calculations were carried out using the 'MEGA' PC program (Kumar *et al.*, 1993).

(Continued overleaf...)



Selected sequences were aligned by 'Pileup' included in the GCG software package (release 8.0), setting gap weights to 3.0 and gap length weights to 0.1 Sequences were extracted from the GenBank database (accession numbers given between brackets), or from recent publications:

F: Phocid distemper virus-2 (PDV-2, L07075), canine distemper virus vaccine strain Onderstepoort (CDV-Vac, X65509), phocid distemper virus-1 (PDV-1, D10371), rinderpest virus (RPV, Z30700), measles virus (MV, U03655), dolphin morbillivirus (DMV, Z30086), porpoise morbillivirus (PMV, X80757).

P_f: The aligned fragments correspond to positions 420 to 807 (388 bases) of the measles virus P gene mRNA. CDV-Vac (X51869), PDV-2 (Mamaev *et al.*, 1996), CDV isolated from a lion of the Tanzanian Serengeti National Park (Harder *et al.*, 1995), CDV isolated from a captive Chinese leopard from the USA (Harder *et al.*, 1996b), PDV-1 (D10371), MV (M89920), RPV (X68311), and DMV and PMV (Barrett *et al.*, 1993).

Viruses which naturally infect marine mammals are indicated by bold typeface.

were closely related members of the genus, but quite different from the other members. They form a distinct lineage more closely related to the ruminant morbilliviruses (RPV and peste-des-petits-ruminants virus, PPRV) than to the carnivore morbilliviruses (Barrett *et al.*, 1993).

Phylogenetic trees of the presently known morbilliviruses, based on sequence analysis of the F genes and this P gene region, show the phylogenetic relations of the newly discovered viruses with the established members of the genus (Fig. 1). Experimental infection studies showed that not only dogs, but also ruminants (cows, sheep and goats) were susceptible to infection with DMV and PMV, which caused a mild leukopenia (Visser *et al.*, 1993b). Pre-exposure to DMV or PMV protected dogs from developing CDV viraemia and clinical signs upon fatal CDV challenge (Visser *et al.*, 1993b). It was speculated as unlikely that DMV or PMV would infect ruminants or dogs under natural circumstances, since morbillivirus infections generally seem to be restricted to one order of mammalian species (Black, 1991). It is interesting to note that in recent years, CDV has been found in many carnivorous species, including seals (Grachev *et al.*, 1989; Osterhaus *et al.*, 1989b), javelinas (Appel *et al.*, 1991), and large cats both in zoos (Appel *et al.*, 1994), and in the wild (Harder *et al.*, 1995).

Origin and epizootiology of the newly recognized morbilliviruses

The origin of PDV-1 was the subject of extensive speculation during and after the outbreak in 1988. Initial speculation about a possible link with CDV infections in sledge dogs in Greenland, or with the PDV-2 (CDV) outbreak among seals in Siberia, could readily be dismissed after the characterization of the virus as a new member of the genus. However, interspecies transfer of the virus is still considered the most likely cause of the outbreak. In sero-epizootiological studies, the presence of a virus closely related, if not identical, to PDV-1 was shown in several North American marine mammal populations (Duignan *et al.*, 1993, 1994; Ross *et al.*, 1992). The virus could have been transmitted by Arctic harp seals (*Phoca groenlandica*), a species that is in contact with both the North American and European seal populations and has been shown to be infected with a PDV-like virus (Dietz *et al.*, 1989; Stuen *et al.*, 1994). Interestingly, a mass migration of harp seals southwards to continental European fishing waters was observed in the period directly preceding the 1988 outbreak. Since virus characterization studies showed that PDV-2, the virus that was isolated from Baikal seals, should be considered as a strain of CDV, the origin of this virus was probably a local terrestrial carnivore. Speculation that the origin might have been a vaccine strain of CDV used to vaccinate local dogs had to be dismissed since nucleotide sequences of PDV-2 P genes showed a closer relationship to European wild type strains than to the vaccine strain used in that area (Mamaev *et al.*, 1996). A similar event may have taken place in the Antarctic region in 1955, when a mass mortality among crabeater seals (*Lobodon carcinophagus*) was possibly related to a simultaneous distemper outbreak among sledge dogs. Support for this assumption came from serological studies showing the presence of CDV neutralizing antibodies in these

animals (Bengtson *et al.*, 1991).

Serological studies have shown that morbilliviruses similar to DMV and PMV are enzootic among many cetacean populations (Visser *et al.*, 1993b). Recently, retrospective immunohistochemical studies suggested the involvement of a morbillivirus in an epizootic among bottlenose dolphins (*Tursiops truncatus*) along the U.S. Atlanti coast in 1987 (Lipscomb *et al.*, 1994). Based on the phylogenetic distinctness from other morbilliviruses, it may be concluded that it is unlikely that the PMV/DMV lineage has a recent origin in terrestrial animals (see Fig. 1). Interestingly, the phylogenetic relationship between the different morbilliviruses of aquatic mammals largely parallels the phylogenetic relationship of the animal species they infect (Visser *et al.*, 1993c). Common viral ancestors may have acquired access to phylogenetically related animal species on the basis of biological similarities: CDV and PDV-1 probably share a common ancestor, which is also likely to be the case for the PMV/DMV and the RPV/PPRV lineages.

Involvement of environmental pollution

Morbilliviruses have probably been enzootic in pinniped and cetacean species for a long time. In general, when these viruses have not been present in a certain population for a long period and consequently no specific immunity is present, an introduction by interspecies transmission may lead to severe outbreaks accompanied by mass mortalities (Norrby *et al.*, 1992; Norrby & Oxman, 1990). However, other factors may have played a role in the recently observed incidence of mass mortalities among marine mammals.

Studies in laboratory animals have shown that exposure to certain environmental contaminants may lead to increased suceptibility to infectious agents (Vos *et al.*, 1989; Wong *et al.*, 1993). The mammalian immune system has long been recognized as a sensitive target for persistent chemicals accumulating in the food chain, including polychlorinated biphenyls (PCBs), -dibenzo-p-dioxins (PCDDs) and -dibenzofurans (PCDFs), hexachlorobenzene (HCB), dieldrin, -hexachloro-cyclohexane (-HCH), and dichlorodiphenyl-trichloroethane (DDT). As top predators, seals and dolphins inhabiting coastal waters of industrialized regions are known to accumulate high levels of some of these xenobiotics (Luckas *et al.*, 1990; Tanabe *et al.*, 1983), and may therefore be at particular risk. These high contaminant burdens have been associated with several physiological abnormalities, including skeletal deformations (Bergman *et al.* 1992; Mortensen *et al.*, 1992), reproduction toxicity (Helle *et al.*, 1976a, 1976b; Reijnders, 1986) and hormonal alterations (Brouwer *et al.*, 1989, Subramanian *et al.*, 1987). While high levels of xenobiotics in affected animals suggest that contaminant-induced immunosuppression may have affected the severity and extent of the morbillivirus epizootics (Aguilar & Borrell, 1994; Hall *et al.*, 1992), direct cause-effect relationship could be established.

We have addressed this issue in a controlled experiment, in which young harbour seals were fed fish contaminated through the food chain of different marine regions. During a 2.5 year period, two groups of 11 harbour seals each were fed herring from the heavily polluted Baltic Sea or the relatively uncontaminated Atlantic Ocean. Intakes of contaminants were three to

ten times higher in seals fed Baltic herring (De Swart *et al.*, 1994), which led to significantly higher blubber levels in this group of seals after two years on the respective diets. During the feeding study, blood samples were taken at regular intervals, and functional immunological parameters were compared. Results demonstrated an impairment of immune function in the seals fed on polluted Baltic herring (De Swart *et al.*, 1994), as evidenced by suppressed natural killer (NK) cell activity (Ross *et al.*, 1996) and suppressed T lymphocyte-mediated responses (De Swart *et al.*, 1996; Ross *et al.*, 1995). Since NK cells play an important role in the first line of defence against virus infections (Welsh & Vargas-Cortes, 1992), and T lymphocytes are important in the clearance of virus infections in general and morbillivirus infections in particular (Van Binnendijk *et al.*, 1990), we have postulated that environmental contaminants rendered seals inhabiting certain polluted areas more susceptible to morbillivirus infections, and may thus have affected the severity and extent of the disease outbreaks. In addition, other possible secondary factors including changes in climate, population densities, food stocks and migration patterns may also have played a role in the outcome of the epizootics.

Conclusions

In the last decade, a seemingly high incidence of previously unrecognized morbillivirus infections among marine mammal populations has been unraveled. This may in part be attributed to the increased interest in the health status of marine mammals, but may also be related to an increased susceptibility of marine mammals to virus infections resulting from environmental contaminant related immunosuppression.

Section 4.3

Canine distemper virus — a morbillivirus in search of new hosts?

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Abstract — Canine distemper morbillivirus (CDV) induces a multisystemic, often fatal disease in a wide and seemingly expanding host range among the *Carnivora*. Several genotypes of an otherwise monotypic virus species co-circulate in a geographically restricted pattern. Interspecies transmissions frequently occur, often leading to devastating epizootics in highly susceptible or immunologically naive populations.

Introduction

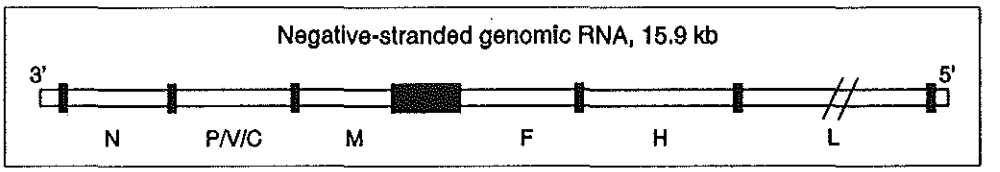
Canine distemper virus (CDV) is a negative-stranded enveloped RNA morbillivirus (*Paramyxoviridae*) that infects a broad host spectrum among the *Carnivora*. This genus also includes the human pathogen, measles virus (MV), rinderpest virus (RPV) and peste des petits-ruminants virus (PPRV) of which the two latter infect *Artiodactyla*. New members of the genus, phocid distemper virus (PDV) and cetacean morbilliviruses, caused widespread severe epizootics among pinnipeds 1988 in northwestern European waters and among toothed cetaceans 1990 - 1991 in the Mediterreanean, respectively (Osterhaus *et al.*, 1995; Visser *et al.*, 1993a). The architecture and molecular biology of morbilliviruses are discussed in Box 1.

Like all morbilliviruses, CDV is highly contagious and transmission occurs predominantly via aerosols. In susceptible hosts, acute febrile and multisystemic disease is induced (Krakowka *et al.*, 1985); neuroinvasiveness and severe immunosuppression are hallmarks of CDV infections (Krakowka *et al.*, 1980; Vandevelde & Zurbriggen, 1995). Depending on the host species and the immune competence of the individual affected, mortality rates during CDV outbreaks can exceed 80% .

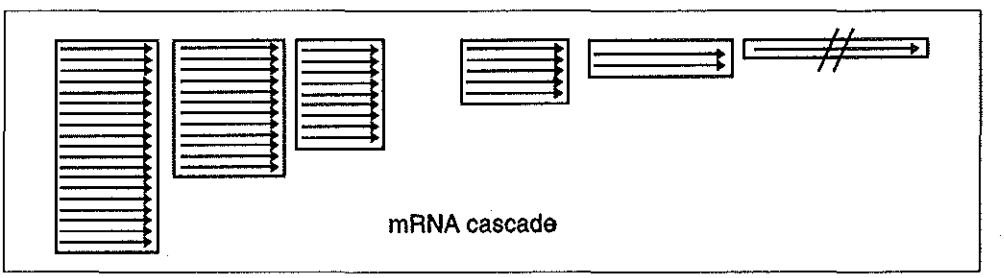
It appears that since 1988, an expansion of the already broad natural host spectrum of CDV has occured. Spontaneous, clinically overt infections with CDV-like morbilliviruses have been described in captive Japanese primates (*Macaca fuscata*; Yoshikawa *et al.*, 1989), collared peccaries (*Tayassu tajacu*; Appel *et al.*, 1991), and Lake Baikal seals (*Phoca sibirica*; Grachev *et al.*, 1989). Furthermore, distemper enzootics caused by CDV-like morbilliviruses have been observed for the first time in lions, tigers and jaguars kept in several North American zoos and safari parks (Appel *et al.*, 1994). In 1994, similar CDV-related disease outbreaks killed up to 30% of the free-ranging lion population and an unknown number of hyaenas in the Tanzanian Serengeti National Park and adjacent areas (Alexander *et al.*, 1995; Harder *et al.*, 1995, 1996b; Haas *et al.*, 1996; Roelke-Parker *et al.*, 1996). Among the *Felidae*, domestic cats are known to be susceptible to experimental CDV infection, but evidence for natural infections, clinical disease or shedding of infectious virus have never been observed (Appel *et al.*, 1974). There is evidence for the involvement of CDV in Paget's disease of humans (Gordon *et al.*, 1992). In addition to an apparently expanded host range of CDV, a resurgence of distemper among dog populations, in which high vaccination rates were maintained, was observed since the late 1980's culminating in major epizootics in France, Germany and Scandinavia during 1991 - 1995 (Blixenkrone-Møller *et al.*, 1993).

Together, these observations raised questions about the occurence of variants of CDV which have allowed expansion into new hosts and challenge the suitability of current CDV vaccines.

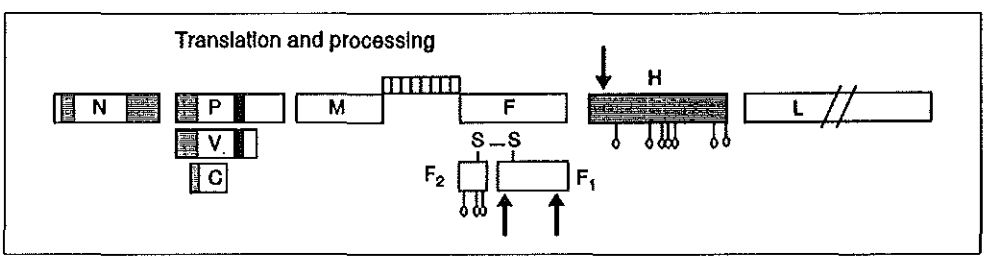
Box 1. Molecular virology of morbilliviruses



Morbillivirions consist of a single-stranded RNA genome of negative polarity (ca. 15900 bp) which is enclosed in a rod-shaped helical nucleocapsid (NC). Six transcription units, from which monocistronic mRNAs are generated, are ordered consecutively along the genome. The entry site of the virion polymerase appears to reside in the 3' leader sequence. At each intergenic region (black boxes) disintegration of the polymerase-RNA complex may occur.



Consequently, a steep gradient of mRNAs from the N gene (most abundant) to the L gene (least abundant) is created. Exceptionally, the P gene also encodes two nonstructural proteins, termed C and V, whose functions are largely unknown.



Regions within the morbillivirus genome that have proven useful in the analysis of strain-dependent variation in MV and other morbilliviruses are shown as grey boxes in this figure. The long intercistronic region between the M and F genes (hatched box) shows a high degree of plasticity between strains but is non-coding and, because of its strong secondary structures, appears difficult to sequence (Rima *et al.*, 1995a). A 388 bp fragment of the P gene (small black box in figure), which can be PCR-amplified from any morbillivirus species using so-called 'universal morbillivirus primers' has been shown to be highly informative for phylogenetic and taxonomic purposes (Barrett *et al.*, 1993). The NC is mainly formed by the N protein, but also contains copies of the polymerase-associated protein (P) and the multifunctional RNA- dependent RNA virion polymerase (L). The NC is enveloped by a host cell-derived lipid layer in which the viral attachment (H) and fusion (F) glycoproteins are embedded. In addition to glycosylation, the F protein undergoes endoproteolytic

Biological and antigenic variants of CDV

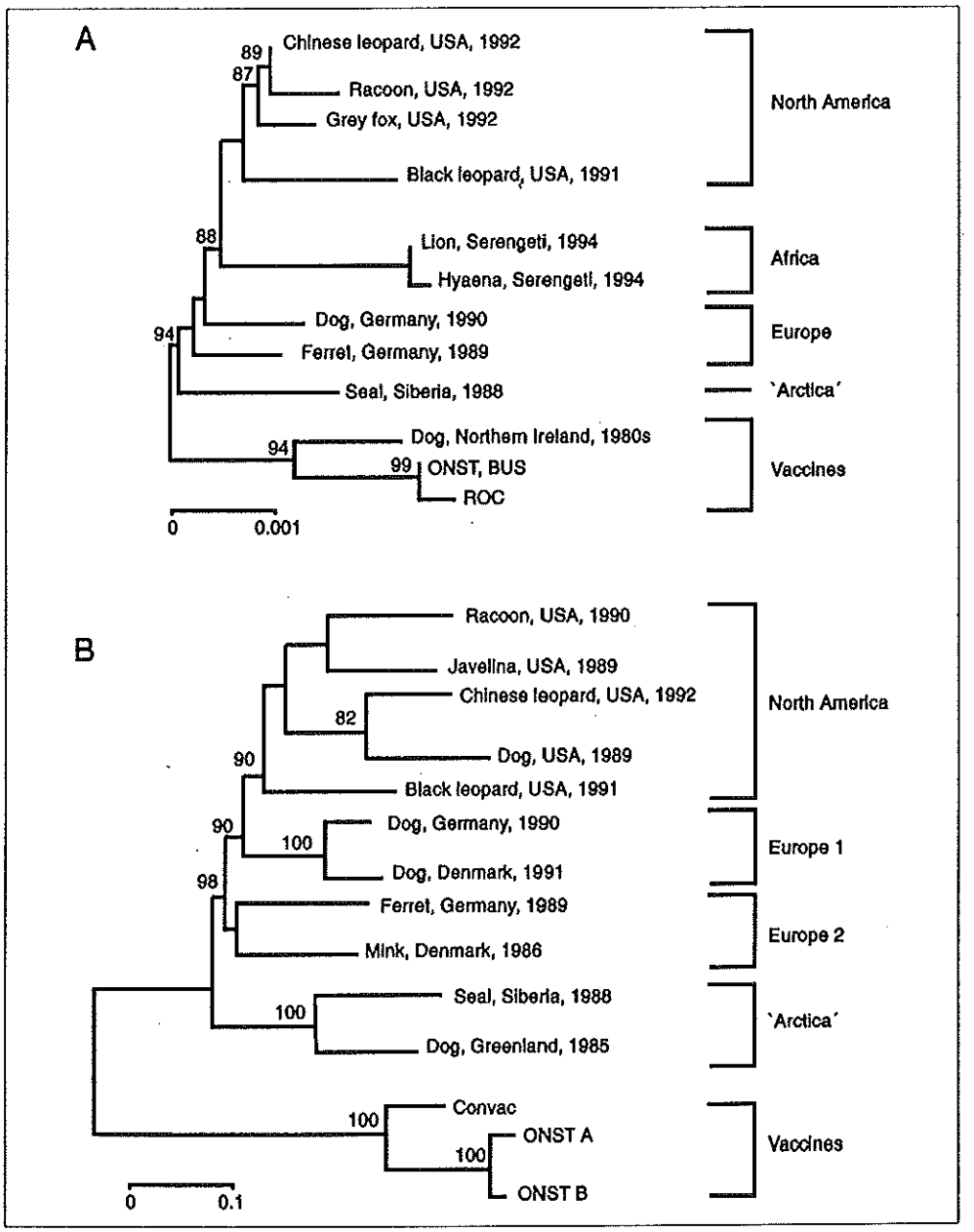
Efficacious modified CDV-live vaccines became available in the 1950s and have been widely used since the 1960s, leading to a drastically reduced impact of distemper on dog populations (Gorham, 1966). Owing to the considerable degree of antigenic relationship between the different morbillivirus species, partial cross-protection against clinically overt CDV infections can also be induced in dogs following heterotypic vaccination with MV (Baker, 1970; Chalmers & Baxendale, 1994). Current licensed CDV vaccines are based on strains that have been attenuated by serial passage either (Rockborn strain; Rockborn, 1959) or on hen eggs (original Onderstepoort strain; Haig, 1956) and chicken fibroblast cultures (Lederle strain). Rockborn strain-based vaccines were shown to induce a higher level of virus neutralizing antibodies compared with Onderstepoort derivates, and consequently avianized CDV vaccines are reported to cause higher vaccine failure rates. The Rockborn strain, however, retains a certain pathogenic potential, particularly for wild species (Appel & Summers, 1995). Although generally safe for use in healthy and immunocompetent dogs, both vaccine types remain extremely virulent for several carnivorous wild species like red pandas (*Ailurus fulgens*), black-footed ferrets (*Mustela nigripes*) or African wild dogs (*Lycaon pictus*), emphazising the species-related differences in susceptibility to CDV (Appel & Summers, 1995). Although virulent and attenuated CDV biotypes can be distinguished by their ability to replicate in various macrophage, lymphocyte and epithelial cell cultures, other *in vitro* markers that differentiate unambiguously between these biotypes have not been defined. Vaccine strains replicate in all these cell types whereas virulent isolates require adaptation (often several blind passages) to replicate efficiently in epithelial cell lines such as Vero cells (Evans *et al.*, 1991; Appel *et al.*, 1992). Among virulent CDV a certain degree of neuroinvasiveness appears to be strain-dependent and has been reported to correlate with plaque morphology in epithelial cell cultures (Hirayama *et al.*, 1986; Summers *et al.*, 1984). The molecular basis of morbillivirus virulence is unknown. In the case of CDV and RPV infections at least, as yet undefined host species-specific, and possibly even breed-specific (RPV), factors also play an important role (Krakowka *et al.*, 1985).

Serologically, all morbillivirus species are considered monotypic and the classical methods, including, for example, complement- fixation and immunofluorescence staining with polyclonal antibodies, do not distinguish serotypes among CDV strains. Nevertheless, vaccine and wild-type (wt) CDV can be differentiated in cross-neutralization and kinetic neutralization assays (Appel *et al.*, 1994; Harder *et al.*, 1993b, 1996b). Sera raised against wt CDV isolates have been shown to have neutralization titers against the homologous virus that are up to tenfold greater than against vaccine strains of CDV (Harder *et al.*, 1996b). Comparable serological results have been obtained for current MV wt isolates and vaccine strains (Tamin

Box 1. – continued:

cleavage into two disulfide-linked fragments (F_1-F_2), which is critical for functional activation. The arrows mark stretches of hydrophobic amino acids capable of spanning membranes. An internal layer of the matrix (M) protein stabilizes the membrane and mediates contact with the NC.

Box 2. Phylogenetic analysis of CDV strains.



et al., 1994). It is the membrane glycoprotein H, the viral attachment factor, that induces the majority of neutralizing antibodies. Consequently, the observed differences in cross-neutralization titers should relate to antigenic variation in the H protein. However, it is difficult to differentiate strains using monoclonal antibodies (MAbs) specific for the CDV H protein. So far, only one epitope has been identified on the H protein that is consistently expressed by CDV vaccine strains but not by most of the recent CDV wt isolates (Blixenkrone-Møller *et al.*, 1992; Alldinger *et al.*, 1993; Appel *et al.*, 1994; Harder *et al.*, 1993b, 1996b). Variation has been observed in the apparent molecular weight of the CDV H protein: the H protein of the Onderstepoort strain migrates significantly faster than those of the Convac and Rockborn vaccine strains (Örvell, 1980) or of various field isolates (Harder *et al.*, 1996b). The lack of a demonstration of more significant antigenic heterogeneity amongst CDV strains (including of other viral proteins such as N, P and F) may, at least in part, relate to the fact that all the CDV- specific MAbs used in these studies were raised against vaccine strains (Örvell *et al.*, 1985).

Box 2. – continued:

Phylogenetic relationships between canine distemper virus (CDV) isolates are based on (a) analysis of a 388 bp fragment of the P gene and (b) on the entire coding region of the H gene (1821 bp). Genetic distances (see bars for scale), calculated by Kimura's 2-parameter method, have been used for tree construction employing the neighbour-joining principle. Identical topologies were obtained when sequences were analysed by maximum parsimony (not shown). Bootstrap values after 500 replications are given at tree branchings only when they exceed 80%. Significant cluster separation is assumed for values $\geq$ 95%. Computations were carried out using the MEGA PC software (Kumar *et al.*, 1994). Sequences were extracted from the GenBank database and recent publications (Harder *et al.*, 1995, 1996; Haas *et al.*, 1996; Mamaev *et al.*, 1995, 1996) Host species, origin and year of isolation are indicated. ONST, Onderstepoort; BUS, Bussell; ROC, Rockborn vaccines.

When sequences of the respective P gene fragment of CDV strains are used in phylogenetic analysis, a clustering of isolates becomes evident, which reflects geographic origin rather than host origin (a). A refined picture of the phylogenetic relationships among CDV strains is obtained when sequences of the gene encoding the the H protein (1821 bp) are analysed (b). The clusters obtained from phylogenetic analysis of the H gene and the P gene fragment are very similar which renders recombination events between the lineages unlikely. At least four to five separate clusters of wt CDV can be distinguished, which differ by more than 0.5% at the nucleotide level (H gene). According to the definition used in analysis of MV strain variation (Rota *et al.*, 1996), these clusters are referred to as genotypes. The vaccine strains form a separate distinct lineage. At the amino acid level the greatest difference is observed between the H proteins of the Onderstepoort vaccine strain and a wt isolate obtained from a Chinese leopard (10.2%). Wt CDV isolates express up to four additional putative N-glycosylation sites located in the extracytoplasmic domaine of the H-protein when compared with the Onderstepoort vaccine strain (Harder *et al.*, 1996; Mamaev *et al.*, 1995).

Even after prolonged serial passage *in vitro*, attenuated strains of MV and RPV accumulate surprisingly few mutations within the coding regions compared with their virulent parents (Baron *et al.*, 1995; Rima *et al.*, 1995a; Rota *et al.*, 1996). Consequently, it can be assumed that the differences between CDV vaccine strains (which originate from wt isolates made in the 1940s and 1950s) and modern wt CDV are truely strain- dependent and do not reflect virulence variations.

Molecular epidemiology of CDV

Genetic analysis of a 388 bp P-gene fragment and of the entire H- protein encoding region shows that several genotypes of wt CDV circulate in a geographically restricted pattern (Box 2). In Europe, at least three different lineages co- circulated between 1989 and 1992. The CDV isolated from big cats of North American or African origin does not constitute a separate 'felid' distemper virus lineage but resembles the CDV circulating in local feral non-felid carnivores. Therefore, the big cat epizootics are likely to be caused by cross-species transmission of CDV indigenous to local wildlife or domestic carnivores (Harder *et al.*, 1996). It remains an enigma why CDV-related epizootics in these species have not occurred earlier or have not been noticed. Possibly, aggravating copathogens or predisposing genetic factors in these particular populations may have played a role and definitely require closer investigation (Roelke-Parker *et al.*, 1996).

Within a group of four CDV isolates from European sources, those of canine origin can be distinguished from those in mustelids ranging in the same area (Box 2). Differences between the 'canine' and the 'mustelid' lineage are on average 3.4% and 4.2% at the nucleotide and the amino acid levels (H-gene and protein, respectively). This substantiates the assumption that these lineages have been co-circulating separately in this area for some time. However, further isolates of canine or mustelid origin from this area have not yet been analysed and interspecies transmissions between these lineages can not be excluded. Therefore, any conclusions about the existance of CDV lineages with host species (family) restriction in this area, which would also implicate that the observed differences indeed reflect a certain advantage to replicating in either mustelid or canine hosts, would be rash.

An intriguing finding is the clustering of an isolate from a distemper-diseased dog (from Belfast, Northern Ireland) with vaccine CDV. Provided that laboratory contamination can be excluded, this case suggests that the comparatively old lineage from which all the vaccine strains have been derived has survived in the field for more than 40 years.

Conclusions

Considering the scarcity of data, the molecular epidemiology of CDV appears to be similar to that of RPV (Chamberlain *et al.*, 1993), in that genetic variants form geographical clusters of serologically monotypic viruses. In MV, up to eight genotypes have been distinguished so far, of which at least four appear to co- circulate globally (Rima *et al.*, 1995a, b). Genetic drift with a linear accumulation of mutations has been demonstrated for the N, P, M, F and H genes of MV (Rima *et al.*, 1995a; Rota *et al.*, 1992, 1995). and also for the H gene of currently circulating wt CDV. All outbreaks of morbillivirus-related disease in species previously not listed as natural hosts of CDV are caused by strains deeply rooted in the CDV cluster, favouring a scenario of interspecies transmission of CDV from local feral carnivores to large felids in the USA and in East Africa.

Antigenic drift in currently circulating wt CDV should be considered a possible factor of a resurgence of distemper cases in well-vaccinated dog populations in Europe. The role of

additional putative N-glycosylation sites predicted in the H proteins of modern wt CDV needs to be elucidated (Mamaev *et al.*, 1995; Harder *et al.*, 1996). Serological evidence indicates that the predicted amino acid changes accumulated in the H protein of circulating wt CDV have antigenic and probably immunological implications (Harder *et al.*, 1996), similar to the MV situation (Tamin *et al.*, 1994). In light of the fact that even heterotypic MV vaccination induces at least partial protection against challenge with virulent CDV in dogs (Baker, 1970; Appel & Gillespie, 1972; Chalmers & Baxendale, 1994), it seems unlikely that modern virulent CDV strains are capable of breaking through a solid immunity mounted after vaccination with either of the common CDV vaccine strains. Nevertheless, epidemiologically, the situation may appear different when facing dog populations with critical CDV-specific herd immunity resulting from low vaccination rates or possibly from frequent use of avianized vaccine derivates which have been reported to be less efficient in inducing immunity as compared to canine cell culture-adapted vaccine strains (Appel & Summers, 1995). Maintenance of high vaccination rates using efficacious vaccines which induce a solid, resilient immunity must still be given the highest priority in control of distemper, particularly in areas with high densities of dogs and their possible exposure to feral carnivores.

Recent morbillivirus epizootics have demonstrated that small populations of highly endangered species may well be at risk of extinction when affected. However, efforts aimed at the protection of such populations, such as the African wild dog (*Lycaon pictus*), by vaccination with proven safe, non-replicating vaccines need to be weighted meticulously against the negative impacts resulting from trapping and immobilizing free-ranging wildlife animals.

Enlarging the collection of CDV-specific sequences obtained globally from a growing number of host species would provide the basis not only for understanding the molecular epidemiology of CDV but also for the improvement of current CDV vaccines. In sharp contrast to MV, where humans are the only relevant reservoir (Rota *et al.*, 1995), the broad natural host spectrum and the high likelihood of interspecies transmission of CDV make eradication impossible.

Questions for future research:

- Where are the reservoirs of virulent CDV?
- Is perpetuation of CDV accomplished in low density multi- species populations?
- Is there an antigenic drift in CDV?
- Do commercial CDV vaccine strains protect fully against current wt CDV?
- What is the molecular basis of virulence in CDV?

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Section 4.4

Summary - Samenvatting -Zusammenfassung

Summary

Certain wildlife species, particularly aquatic mammals and the larger carnivorous species have always exerted a strong fascination on man. This may, at least in part, explain the extraordinary public concern about devastating morbillivirus epizootics in 1988, 1990-1991 and 1994 in seals, dolphins and lions, respectively, which not least drew virus infections of these species also into a scientific focus. The **intention of this thesis** is to broaden the knowledge of morbilli- and herpesvirus infections of aquatic and terrestrial carnivores aiming also at a contribution to an improved protection of these partly endangered species. A special emphasize is put on the phylogenetic and epidemiological relationships of morbilli- and herpesviruses, respectively, of these species.

Part I summarizes molecular and clinical studies on *herpesvirus infections in pinnipeds*:

Seals are host to at least two species of herpesviruses, referred to as phocid herpesvirus-1 and -2 (PhHV-1, -2), which can be readily distinguished by means of their biological properties in cell culture, by cross neutralization, and by typing with monoclonal antibodies. PhHV-1 has been molecularly identified as a distinct member of the *Varicellovirus* genus of the α–*Herpesvirinae* subfamily. In antigenetic and phylogenetic terms, the authentic herpesviruses of terrestrial carnivores, canid and felid herpesvirus, were found to represent the closest relatives of PhHV-1. PhHV-1 or other closely related α–herpesviruses appeared to be prevalent on a globally in all pinniped populations examined so far. The clinical impact of primary PhHV-1 infections, shown to be inversely correlated to the age and immunocompetence of the affected individual seal, is considered to be significant and ranged from mild upper respiratory disorders to bronchopneumonia and fatal generalized exacerbations. An improved diagnosis of active PhHV-1 infection was enabled by a novel PCR assay targeting a fragment of the gene encoding the PhHV-1 glycoprotein D equivalent. The glycoprotein B homologue of PhHV-1 is shown to be a major immunogenic protein in the course of natural infections of seals. The gene encoding gB has been sequenced in completion and its product is successfully expressed as an antigenically and immunogenically authentic molecule in insect cells. Recombinant gB may serve as a major component of a future PhHV-1 subunit vaccine.

Described as a novel viral agent of pinnipeds for the first time in this thesis, PhHV-2 is molecularly characterized as a member of the γ–*Herpesvirinae*. Phylogenetic analysis of a fragment of the major capsid protein suggested its classification, together with the equine herpesvirus-2, in a third genus of this subfamily. As yet, no disease conditions have been associated with PhHV-2 infections in pinnipeds although, based on serosurveys, the virus appeared to be moderately prevalent in free-ranging pinniped populations.

Both PhHV-1 and -2 replicated efficiently in cell cultures of feline origin. The possibility of a natural interspecies transmission of these viruses to members of the *Felidae* family cannot be ruled out at present.

In **Part II** of this thesis, the identity and the relationships of *morbilliviruses* which have caused devastating epizootics *in pinnipeds* (1988, northwestern European waters) *and in terrestrial carnivores* (1994, Serengeti National Park, Tanzania) are investigated:

Canine distemper virus (CDV) was found to be associated with recent losses among big cats (tigers, leopards and lions) in North American zoos (1991 - 1993) and with a major outbreak affecting the lion population of the Tanzanian Serengeti National Park (1994). Phylogenetic analysis of the viruses involved did not support the assumption of an emerging 'felid distemper' virus, but revealed that the outbreaks among big cats were caused by several distinct yet true CDV strains. Since these strains were also found to circulate in local feral carnivores, interspecies transmission of 'indigenous', geographically restricted CDV strains appeared to be the most likely source of the CDV epizootics in big cats. In contrast, the seal mass mortality in northwestern European waters has been caused by a distinct member of the morbillivirus genus, the phocid distemper virus (PDV). While the available PDV strains appeared to be antigenically homogeneous, genetic drift, and, by circumstantial evidence, possibly also antigenic drift, have occured in CDV strains which could have played a role also in the recent resurgence of distemper in domestic dog populations.

In **conclusion**, two distinct herpesvirus species of pinnipeds were identified and molecularly characterized. Promising prerequisites for a subunit vaccine aiming at the protection of hospitalized seals (e.g. rehabilitation, breeding programmes) against clinical disorders associated with the pathogenic PhHV-1 α—herpesvirus are provided. In addition, the molecular epidemiology and the phylogenetic relationships of morbilliviruses which caused recent epizootics among terrestrial wildlife carnivores were partly elucidated, revealing a co-circulation of distinct contemporary CDV genotypes which appeared to be restricted geographically and, thus, not species-specifically. It therefore seemed unlikely that the apparent expansion of the natural host spectrum of CDV, now comprising also pinnipeds and exotic felids, could have been due to an emerging CDV type exhibiting an altered host tropism.

Samenvatting

Wilde dieren, in het bijzonder zeezoogdieren en grote op het land levende roofdieren, hebben altijd een bijzondere aantrekkingskracht op de mens uitgeoefened. Het is dan ook niet verwonderlijk dat de dramatische uitbraken van morbillivirusinfecties onder zeehonden (1988), dolfijnen (1990-1991) en leeuwen (1994) grote publieke aandacht hebben getrokken en hernieuwde wetenschappelijke belangstelling hebben gewekt voor virusinfecties in deze diersoorten.

Dit proofschrift beoogde het inzicht op het gebied van morbillivirusinfecties en herpesvirusinfecties in zeezoogdieren en op het land levende vleeseters te vergroten en aldus een bijdrage te leveren aan de bescherming van deze diersoorten, waarvan er verschillende met uitsterven worden bedreigd.

In **deel I** van dit proofschrift werden moleculaire en klinische studies beschreven aangaande herpesvirusinfecties in vinpotigen, een suborder van vleesetende zeezoogdieren waartoe zeehonden en walrussen behoren. In zeehonden kunnen tenminste twee sorten herpesvirussen voorkomen: phocid herpesvirus-1 en -2 (PhHV-1, -2). Deze twee virussen kunnen van elkaar worden onderscheiden op grond van hun biologische eigenschappen in celkweek, door kruisneutralisatie en door typering met monoklonale antistoffen. Op moleculair niveau werd PhHV-1 geïdentificeerd als een apart lid van het *Varicellovirus* geslacht, behoren tot de subfamilie van α–*Herpesvirinae*. De oorspronkelijke herpesvirussen van op het land levende vleeseters, de honden- en kattenherpesvirussen, blijken antigenetisch en phylogenetisch het meest verwant te zijn aan PhHV-1. Onderzoek tot op heden heeft aangetoond dat PhHV-1 of andere nauw verwante α–herpesvirussen wereldwijd voorkomen in alle vinpotigen. Aangetoond werd dat de ernst van de klinische symptomen ten gevolge van een primaire infectie met PhHV-1 omgekeerd evenredig is met de leeftijd en het immunologisch reactievermogen van een geïnfecteerd dier en kan varieren van eem milde aandoening van de bovenste luchtwegen, tot een bronchopneumonie en gegeneraliseerde aandoening met fatale afloop. De diagnostiek van een actieve PhHV-1 infectie werd aanzienlijk verbeterd door de ontwikkeling van een nieuwe PCR test, waarbij een fragment werd geamplificeerd van het gen dat codeert voor het equivalent van het glykoproteïne D van PhHV-1. Het glykoproteïne B (gB) homoloog van PhHV-1 bleek het belangrijkste immunogene eiwit in zeehonden die op natuurlijke wijze geïnfecteerd zijn met PhHV-1. Het gen dat voor gB codeert werd volledig gesequenced en het genprodukt tot expressie gebracht in insektencellen als een antigeen en immunogeen eiwit. Dit recombinant gB kan een belangrijk bestanddeel vormen van een toekomstig PhHV-1 subunit vaccin.

PhHV-2, dat in dit proefschrift voor het eerst werd beschreven als een nieuw virus van vinpotigen, is op moleculair niveau geidentificeerd als een lid van de γ–*Herpesvirinae* subfamilie. Phylogenetisch onderzoek van een fragment van het grootste capside eiwit suggereerde dat PhHV-2, samen met het equine herpesvirus-2, behoort tot een derde geslacht van deze subfamilie. Hoewel door serologisch onderzoek werd aangetoond dat PhHV-2, zij het op beperkte schaal, voorkomt bij in het wild levende populaties van vinpotigen, zijn er tot op heden geen ziekteverschijnselen geassocieerd met een PhHV-2 infectie. Zowel PhHV-1 als PhHV-2

vermeerden zich in kattencellen. Dat deze virussen onder natuurlijke omstandigheden ook leden van de familie van de *Felidae* zouden kunnen besmetten kan vooralsnog niet worden uitgesloten.

In **deel II** van dit proefschrift is onderzoek beschreven naar de aard van en de verwantschap tussen morbillivirussen die uitbraken hebben veroorzaakt onder vinpotigen in Noord-West Europese wateren (1988) en onder op het land levende vleeseters in het Serengeti National Park in Tanzania (1994). De recente uitbraken van morbillivirusinfecties onder grote katachtigen in Noord Amerikaanse dierentuinen (1991–1993) en onder leeuwen in het Serengeti National Park in Tanzania (1994) zijn geassocieerd met het voorkomen van verschillende stammen van het canine distemper virus (CDV). Op grond van moleculair onderzoek werd geen bewijs verkregen voor een echte adaptatie van CDV aan katachtigen. Phylogenetisch onderzoek toonde echter aan dat natuurlijke overdracht van CDV afkomstig van andere wilde dieren, maar grote katachtigen zeer waarschijnlijk de belangrijkste factor is in de CDV uitbraken onder grote katachtigen. De massale sterfte onder zeehonden in Noord-Europese wateren, daarentegen, is echter wel veroorzaakt door een apart lid van het morbillivirus geslacht: het phocid distemper virus (PDV). Terwijl de PDV stammen in antigen opzicht homogeen blijken te zijn, hebben er in de CDV stammen genetische, en dientengevolge mogelijk ook antigene, veranderingen plaatsgevonden, die wellicht tevens een rol hebben gespeeld in de recente opleving van hondenziekte in gedomesticeerde honden.

Concluderend kan worden gesteld dat in dit proefschrift twee verschillende soorten herpesvirussen van vinpotigen werden geïdentificeerd en op moleculair niveau gekarakteriseerd. Belangrijke vorderingen werden gemaakt op de weg naar de ontwikkeling van een vaccin dat bescherming zal bieden tegen de ontwikkeling van klinische symptomen, van een PhHV-1 α–herpesvirus infectie. Verder werden de moleculaire epidemiologie en de phylogenetische verwantschappen opgehelderd van morbillivirussen, die recente uitbraken onder wilde landroofdieren hebben veroorzaakt. Deze studies gaven aan dat er verschillende gelijkttijdig circulerende CDV genotypen zijn die een verschillend verspreidingsgebied hebben. Daarom lijkt het onwaarschijnlijk dat de kennelijk opgetreden uitbreiding van het natuurlijk gastheerspectrum van CDV, waartoe nu ook vinpotigen en exotische katachtigen behoren, het gevolg is geweest van het ontstaan van CDV stammen met een veranderd gastheer tropisme.

Zusammenfassung

Bestimmte Wildtierarten, insbesondere aber Meeressäugetiere und größere landlebende Fleischfresser, üben von jeher eine starke Faszination auf Menschen aus. Dies kann zumindest teilweise die enorme öffentliche Anteilnahme an den verheerenden Morbillivirusepizootien in wildlebenden Populationen von Seehunden (1988), Delphinen (1990 - 1991) und Löwen (1994) begründen. Nicht zuletzt das Gewicht dieses öffentlichen Interesses ermöglichte auch eine intensivierte Forschungsarbeit auf dem Gebiet der Virusinfektionen dieser Wildtierarten. Das **Bestreben der vorgelegten Arbeit** gilt der Erweiterung des Wissens über Morbilli- und Herpesvirusinfektionen von Meeressäugern, wie auch landlebenden Fleischfressern, mit dem Ziel, zu einem verbesserten Schutz dieser z.T. gefährdeten Spezies in der Zukunft beizutragen. Ein Akzent dieser Studie liegt auf den phylogenetischen und epidemiologischen Beziehungen der jeweiligen Virusspezies.

Teil I umfaßt Beiträge zu den *Herpesvirusinfektionen der Flossenfüßer*:

Robben stellen Wirte für zumindest zwei Arten von Herpesviren dar, die als phocides Herpesvirus-1 bzw. -2 (PhHV-1, -2) bezeichnet werden und problemlos anhand ihrer biologischen Eigenschaften in Zellkulturen, durch Kreuzneutralisation und mittels monoklonaler Antikörper zu unterscheiden sind. Auf molekularer Ebene konnte PhHV-1 als eigenständige Spezies im Genus *Varicellovirus* der α–*Herpesvirinae*-Unterfamilie klassifiziert werden. Nach antigenetischen und phylogenetischen Gesichtspunkten repräsentieren die genuinen Herpesvirusarten landlebender Fleischfresser (das felide bzw. kanide Herpesvirus) die engsten Verwandten des PhHV-1. Infektionen mit PhHV-1 oder anderen, eng verwandten Varianten sind weltweit verbreitet in allen bislang untersuchten Flossenfüßerpopulationen anzutreffen. Die klinische Bedeutung primärer PhHV-1-Infektionen, die invers mit dem Alter und der Immunkompetenz der jeweils betroffenen Robbe korreliert, muß als beachtlich eingestuft werden und variiert von milden Affekten des oberen Respirationstraktes über Bronchopneumonien bis hin zu tödlich verlaufenden Generalisationen. Die Möglichkeiten der Diagnose einer aktiven PhHV-1 Infektion konnten durch die Entwicklung eines PCR Tests, der auf die spezifische Amplifikation eines Fragments des Glykoprotein-D-Gens abhebt, entscheidend verbessert werden. Das Glykoprotein-B (gB)-Equivalent des PhHV-1 wurde als ein immundominantes virales Protein im Rahmen natürlicher Infektionen von Robben charakterisiert. Das gB-Gen wurde sequenziert und sein Produkt erfolgreich als ein antigenetisch intaktes und immunogenes Molekül in Insektenzellen exprimiert. Somit erscheinen die Voraussetzungen günstig, rekombinantes gB als eine Komponente einer zukünftigen PhHV-1 Subunit-Vakzine einzusetzen.

PhHV-2 wird erstmals im Rahmen dieser Abhandlung als ein neues Virus von Pinnipeda beschrieben und auf molekularer Ebene als γ–Herpesvirus charakterisiert. Die phylogenetische Analyse eines Fragments des Hauptkapsidproteins des PhHV-2 legt dessen Klassifizierung, zusammen mit dem equinen Herpesvirus-2, in einem dritten Genus der γ–Herpesviren nahe. Bislang konnten PhHV-2-Infektionen nicht mit Krankheitserscheinungen bei Robben korreliert werden, obwohl das Virus, basierend auf serologischen Untersuchungen, mit moderater Prävalenz auch in wildlebenden Robbenpopulationen anzutreffen ist.

Sowohl PhHV-1 wie auch PhHV-2 replizieren effizient in Zellkulturen felinen Ursprungs.

Eine etwaige Übertragung phocider Herpesviren unter natürlichen Bedingungen auf Mitglieder der Familie der Feliden kann derzeit nicht ausgeschlossen werden.

Teil II dieser Arbeit befaßt sich mit der Identifizierung und molekular-epidemiologischen Charakterisierung von *Morbilliviren*, die 1988 *bei Robben* in nordwesteuropäischen Gewässern und 1994 bei Löwen *und anderen Fleischfressern* in der Serengeti (Tansania) zu verheerenden Epizootien geführt hatten:

Eine Beteiligung des Hundestaupevirus (CDV) an jüngsten seuchenhaften Todesfällen unter Großkatzen in nordamerikanischen Zoos (1991 - 1993) sowie an einer Epizootie in der Löwenpopulation im tansanischen Serengeti-Nationalpark (1994) konnte nachgewiesen werden. Die phylogenetische Analyse der involvierten Viren ergab keine Anhaltspunkte für die zunächst geäußerte Vermutung, daß es sich hier um eine sich neu entwickelnde CDV-Linie, also um ein 'Katzenstaupevirus' handeln könnte. Im Gegenteil: Die einzelnen Ausbrüche wurden durch verschiedene, jedoch jeweils eindeutig dem Hundestaupevirus zuzuordnende Virusstämme ausgelöst. Da dieselben Stämme auch bei verschiedenen ortsansässigen nicht-feliden Fleischfresserarten angetroffen wurden, erscheint die zwischenartliche Übertragung 'einheimischer', also geographisch begrenzt vorkommender CDV-Stämme als die wahrscheinlichste Quelle der CDV-Epizootien bei Großkatzen. Im Gegensatz hierzu wurde das Robbensterben 1988 durch eine eigenständige Virusspezies des Genus *Morbillivirus* ausgelöst (phocides Staupevirus, PDV). Während die bislang untersuchten PDV-Isolate antigenetisch einheitlich zu sein scheinen, sind CDV-Stämme einer genetischen Drift unterworfen, die sich – aufgrund indirekter Hinweise – auch antigenetisch auswirken und damit eine Rolle in dem seit kurzem beobachteten Wiederaufflackern der Staupe bei Haushunden spielen könnte.

Zusammenfassend bleibt festzuhalten: In dieser Arbeit wurden zwei verschiedenartige, bei Pinnipeda vorkommende Herpesvirusspezies identifiziert und auf molekularer Ebene charakterisiert. Es wurde eine vielversprechende Basis für die Entwicklung einer 'Subunit'-Vakzine erarbeitet, die vor allem hospitalisierte Robben (z.B. Rehabilitation, Arterhaltungsprogramme) vor den klinischen Folgen einer Infektion mit dem pathogenen PhHV-1 α–Herpesvirus schützen soll. Weiterhin konnten molekular-epidemiologische und phylogenetische Zusammenhänge von Morbillivirusinfektionen, die unlängst vehemente Epizootien bei landlebenden Fleischfressern verursacht hatten, zumindest partiell aufgeklärt werden. Hierbei wurde deutlich, daß unterschiedliche CDV-Genotypen kozirkulieren, deren Vorkommen geographisch, also nicht tierartspezifisch beschränkt ist. Es erscheint somit unwahrscheinlich, daß die offenbare Ausweitung des CDV-Wirtsspektrums auf Pinnipeda und Großkatzen auf das Auftreten neuer, bislang unbekannter CDV-Varianten mit einem veränderten Wirtstropismus zurückgeführt werden kann.

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Curriculum vitae

The author of this thesis was born on March 13, 1963, in Rendsburg (Schleswig-Holstein), Germany, where - in 1982 - he took his 'Abitur'. His veterinary medical studies at the Hannover Veterinary School (HVS), Germany, commenced in 1983 and were completed in 1988 (DVM-equivalent). A doctoral thesis on 'Morbillivirus infections in pinnipeds' was prepared at the Institute of Virology, HVS, under supervision of Prof. Dr. B. Liess from 1989 to 1991. The author obtained a doctoral degree from the Hannover Veterinary School in 1991. During the following two years he was affiliated with the Institute of Virology, HVS, as a scientific co-worker. In 1994, in the frame of a human capital and mobility project of the European Union, the author took up a 2-years research position in the laboratory of Prof. Dr. A. Osterhaus, head of the Department of Virology, Erasmus University Rotterdam, The Netherlands. Following employment intermezzi at the Dutch Seal Rehabilitation and Research Center, Pieterburen, and, again, at the HVS Institute of Virology in 1996 (head: Prof. Dr. V. Moennig), the author is now affiliated with the Institute of Medical Microbiology and Virology at the Christian-Albrechts-University, Kiel, Germany (head: Prof. Dr. U. Ullmann).

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[1]Caught with red hands?

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