Characterization of phocid herpesvirus-1 and -2 as putative alpha- and gammaherpesviruses of North American and European pinnipeds

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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 Californian 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 that 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 *Alphaherpesvirinae* and in particular to CHV. Sequence analysis of two *Eco*RI fragments of the PhHV-2 genome (European isolate 7848) revealed greatest similarity to gammaherpesviruses 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 gammaherpesvirus 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 *Alphaherpesvirinae* (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 the seal populations of

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Antarctica and the Northern Pacific (Vedder *et al.*, 1987; Harder *et al.*, 1991; Stenvers *et al.*, 1992).

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-2 genome are provided.

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 2×10^5 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 2 h at 37 °C while rolling. Then cultures were washed twice with PBS and reconstituted with 1 ml of 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 micro-titration 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 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 vols of a buffer containing 50 mM-Tris (pH 8·0), 5 mM-EDTA and 0·5% (v/v) NP40 while incubating for 2 min on ice. Insoluble residues were pelleted and *N*-laurylsarcosinate (NLS; 0·5%, w/v, final concn) as well as Proteinase K (250 μg/ml 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 PCR. 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 limited available sequence data for these viruses (Maeda *et al.*, 1992, 1994; Spatz & 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 herpesviru type 1 (EHV-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 min and consisted of 33 cycles of 30 s at 94 °C, 1 min at 37, 45 or 55 °C and 1 min at 72 °C. A final elongation step at 72 °C was allowed for 10 min. 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 μM of sense and antisense primer and 2·5 U Ampli*Taq* polymerase. $MgCl_2$ was added separately from a stock to give the desired

Table 1. *Origin of herpesviruses*

Virus*	Host [disease]†	Origin	Specimen [permissive cell line]	Reference
PhHV-1				
PB84	*Phoca vitulina* [gen.]	Dutch Wadden Sea	Kidney [CrFK]	Osterhaus *et al.* (1985)
2557/Han88	*Phoca vitulina* [gen.]	German Wadden Sea	Leukocytes [CrFK]	Frey *et al.* (1989)
550/Hel93	*Halichoerus grypus* [gen.]	Baltic Sea, Gulf of Riga	Urinary bladder [CrFK]	This study (Kulonen)‡
A92-10/4	*Phoca vitulina* [−]	Long Island, USA	Lung [CrFK]	This study (Appel)
Ja/PB94	*Phoca vitulina* [resp.]	Dutch Wadden Sea	Nasal swab [CrFK]	This study (Osterhaus)
PhHV-2				
7848/Han90	*Phoca vitulina* [gen.]	German Wadden Sea	Leukocytes [CrFK]	Lebich *et al.* (1994)
Zalo/Cal86	*Zalophus californianus* [gen.]	California, USA	Lung [CrFK]	Kennedy-Stoskopf *et al.* (1986)
A92-10/5	*Phoca vitulina* [−]	Long Island, USA	Lung [CrFK]	This study (Appel)
A93-2/2	*Phoca vitulina* [−]	Long Island, USA	Leukocytes [Vero]	This study (Appel)
A93-2/11	*Phoca vitulina* [−]	Long Island, USA	Leucocytes [Vero]	This study (Appel)
FHV				
6887/Han91	*Felis catus* [resp.]	Germany	Pharyngeal swab [CrFK]	Lebich *et al.* (1994)
CHV				
5105/Han89	*Canis familiaris* [resp.]	Germany	Lung [MDCK]	Harder *et al.* (1991)

* Clustering based on typing with MAbs (see Fig. 2).
† [resp.], respiratory symptoms; [gen.], generalized, fatal disease; [−], no data available.
‡ Virus isolation performed in the laboratory of the author indicated.

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

Designation	Sequence (5′ → 3′)*	Location (CHV)†	Expected product sizes (bp) CHV	FHV‡
gD-1	gAA gTT Cgg TAT gT[A/T*] AC	142 → 158	290	287
gD-2	AAT CCC AAT TCA TC[A/ġ*] TC	415 ← 431		
gB-1	ACȦ ACT gTA Tgg TCT gg	358 → 374	367	367
gB-2	ĊȦC Cgg Tgg AgA TAg C	709 ← 724		
gB-3	gCT ȦCA gTT TgC CTA TgA	1431 → 1448	1616	1632
gB-4	ggT Aġ A AAT TCA CgA TC[C/T*] TC	1954 ← 1973	543	540

* Primers were basically chosen 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 & Maes (1993) and Spatz *et al.* (1994); position 1, translation-initiation ATG.

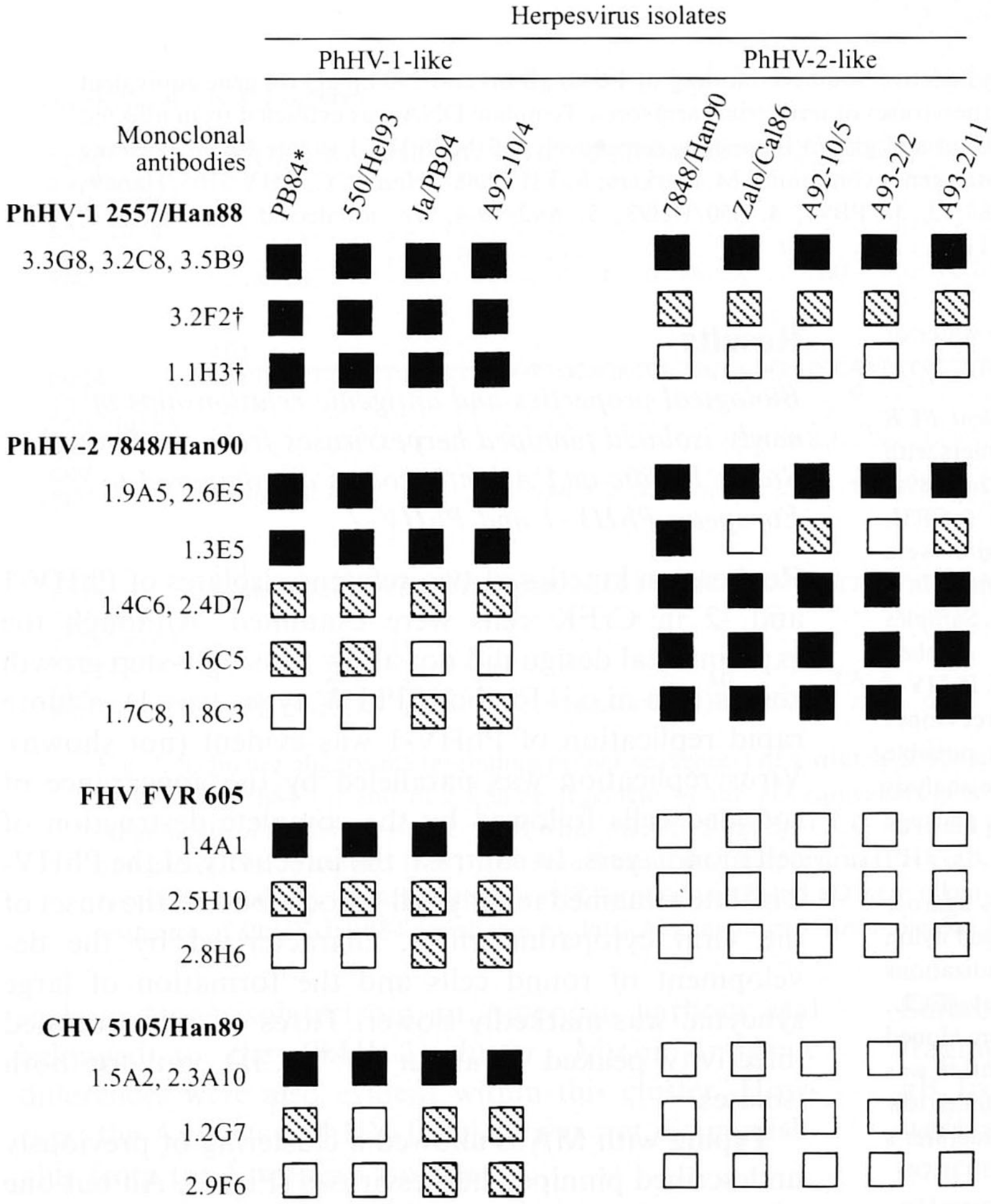

Fig. 1. Reactivities of MAbs raised against various pinniped herpesvirus isolates (given in bold type) analysed by an enzyme immunoassay employing fixed infected CrFK cells. Cross-reactivity gradations were evaluated using arithmetic mean values of at least three titration experiments: strong (■), weak (▧) and not detectable (□) (titre ⩽ 1:10). The differences in titre 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).

concentration. A 5 µl volume of DNA prepared from tissue cultures was added to give a final volume 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 to minimize 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

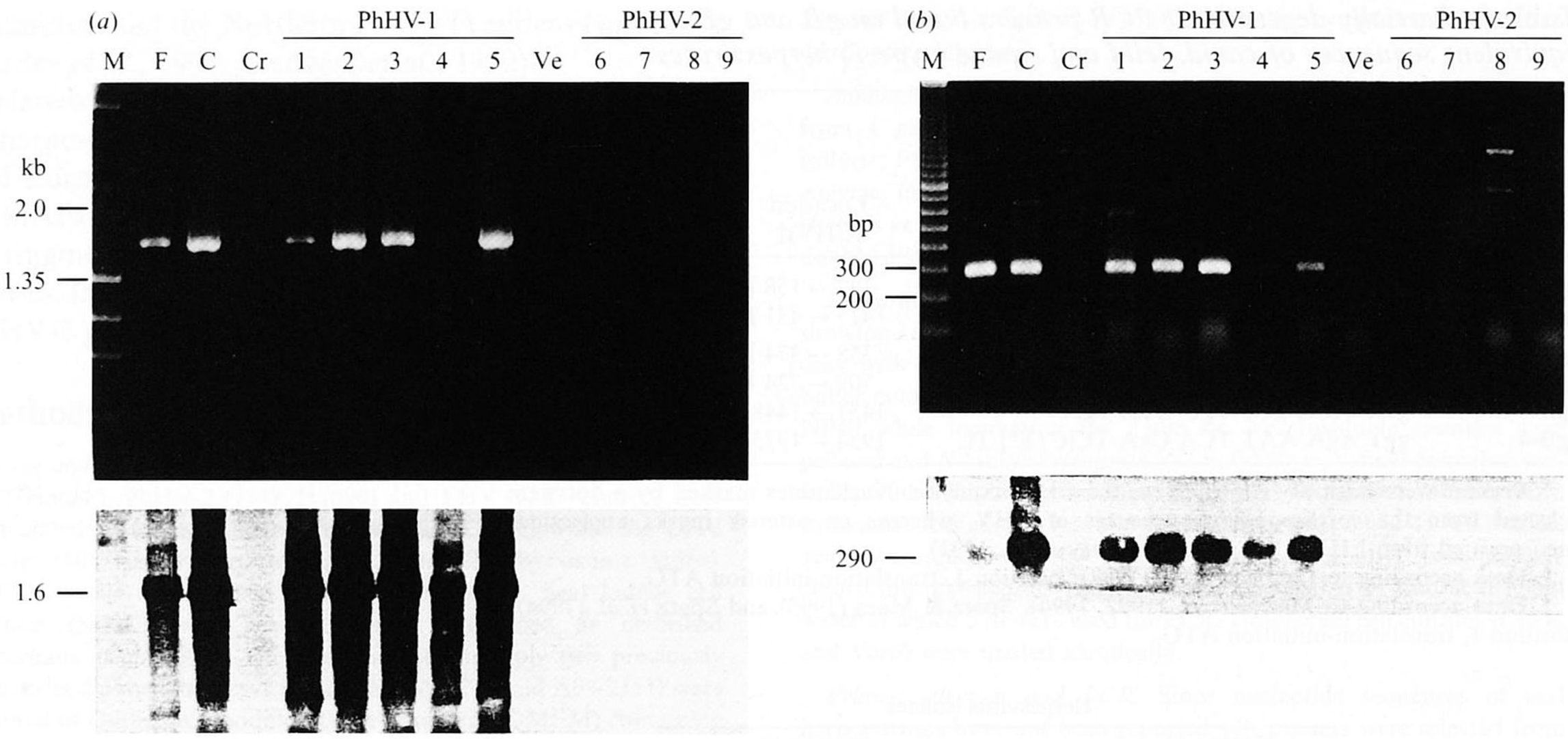

Fig. 2. Amplification by PCR and detection by non-radioactive Southern blotting of 1·6 kb gB (*a*) and 290 bp gD (*b*) gene equivalent fragments from various pinniped herpesviruses and herpesviruses of terrestrial carnivores. Template DNA was extracted from infected cell cultures and probes were prepared 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*) stringency conditions. M, markers; 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.

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 'sticky'-T plasmid vector (pCRII, 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 stringency 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 ECL using full-length virion DNA as a probe. Two clones carrying PhHV-2-specific inserts of 315 and 788 bp were sequenced to 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 PhHV-1 was evident (not shown). Virus replication was paralleled by the 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. Titres of cell-associated infectivity peaked at about $10^{5.0}$ $TCID_{50}$/ml 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

(*a*) *Glycoprotein B fragment*

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                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..
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(*b*) *Glycoprotein D fragment*

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                1                                                                                                  100
PB84            ATCTACAGATCCATGTGGTATGGTTGCTTTAATTTCTGAGCCAAATATAGAATCTACAATTAAAACTATTCAATTTGTAAATAAAAAAAAATATTATAAC
Ja/PB94         ....................................................................................................T
550/Hel93       ....................................................................................................
A92-10/4        ....................................................................G.....GC.A..G.....T...C..T
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        ....................................................T.....C........G...T..........G...A.....C.....T.
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.....
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Fig. 3. Sequence alignments (excluding primer sequences) of a stretch 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' from 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 (–).

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.

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 the 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. 2*a*). Similarly, no

(*a*)

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  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*)

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  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
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Fig. 4. Nucleotide and deduced amino acid sequences of a 788 bp (*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).

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

Table 3. *Comparison of amino acid sequences predicted from two EcoRI fragments of the PhHV-2 isolate 7848*

PhHV-2 fragment	Virus species	Gene equivalent*	Comparison of ORFs† 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	UL70	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

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

† 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).

primer sequences) to gB genes of FHV and CHV confirmed the specificity of the amplificate (Fig. 3*a*). Nucleotide identities of 79% and 75% to CHV and FHV, respectively, were observed in that region.

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 stringency conditions significant hybridization was achieved only with PhHV-1 isolates and CHV (Fig. 2*b*).

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 geographical 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 the corresponding regions of FHV and CHV gD genes is presented in Fig. 3(*b*) 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 bp (*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 gammaherpesviruses (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 gammaherpesviruses 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 gammaherpesvirus 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 alpha- or betaherpesviruses (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).

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 leukocytes (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 degenerate 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 PCR amplification of PhHV-1 gD 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 geographical 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.*, 1991; Stenvers *et al.*, 1992; Lebich *et al.*, 1994).

The taxonomic status of PhHV-2 was elucidated by sequence analysis of two *Eco*RI fragments. A clear clustering with gammaherpesviruses 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 gammaherpesvirus 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 gammaherpesvirus. On the basis of the limited sequence data, the closest relative of PhHV-2 amongst the gammaherpesviruses 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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References

ALBRECHT, J. C., NICHOLAS, J., BILLER, D., CAMERON, K. R., BIESINGER, B., NEWMAN, C., WITTMANN, S., CRAXTON, M., COLEMAN, H., FLECKENSTEIN, B. & HONESS, R. W. (1992). Primary structure of the Herpesvirus saimiri genome. *Journal of Virology* **66**, 5047–5058.

CRUTE, J. J., TSURUMI, T., ZHU, L., WELLER, S. K., OLIVO, P. D., CHALLBERG, M. D., MOCARSKI, E. S. & LEHMAN, I. R. (1989). Herpes simplex virus 1 helicase–primase: a complex of three herpes-encoded gene products. *Proceedings of the National Academy of Science, USA* **86**, 2186–2189.

DAYHOFF, M. O., BARKER, W. C. & HUNT, L. T. (1983). Establishing homologies in protein sequences. *Methods in Enzymology* **91**, 524–545.

DEVEREUX, J., HAEBERLI, P. & SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387–395.

ENGELS, M., GELDERBLOM, H., DARAI, G. & LUDWIG, H. (1983). Goat herpesviruses: biological and physicochemical properties. *Journal of General Virology* **64**, 2237–2247.

FREY, H.-R., LIESS, B., HAAS, L., LEHMANN, H. & MARSCHALL, H. J. (1989). Herpesvirus in harbour seals (*Phoca vitulina*): isolation, partial characterization, and distribution. *Journal of Veterinary Medicine B* **36**, 699–708.

HARDER, T. C., PLÖTZ, J. & LIESS, B. (1991). Antibodies against European phocine herpesvirus isolates detected in sera of Antarctic seals. *Polar Biology* **11**, 509–512.

HORVAT, B., WILLHAUS, T., FREY, H.-R. & LIESS, B. (1989). Herpesvirus in harbour seals (*Phoca vitulina*): transmission in homologous hosts. *Journal of Veterinary Medicine B* **36**, 715–718.

KENNEDY-STOSKOPF, S., STOSKOPF, M., ECKHAUS, A. M. & STRANDBERG, J. D. (1986). Isolation of a retrovirus and a herpesvirus from a captive California sea lion. *Journal of Wildlife Diseases* **22**, 156–164.

LEBICH, M., HARDER, T. C., FREY, H.-R., VISSER, I. K. G., OSTERHAUS, A. D. M. E. & LIESS, B. (1994). Comparative immunological characterization of type-specific and conserved B-cell epitopes of pinniped, felid and canid herpesviruses. *Archives of Virology* **136**, 335–347.

LIMBACH, K. J., PAULLIN LIMBACH, M., CONTE, D. & PAOLETTI, E. (1994). Nucleotide sequence of the genes encoding the canine

herpesvirus gB, gC and gD homologues. *Journal of General Virology* **75**, 2029–2039.

MAEDA, K., HORIMOTO, T., NORIMINE, J., KAWAGUCHI, Y., TOMONAGA, M., NIIKURA, M., KAI, C., TAKAHASHI, E. & MIKAMI, T. (1992). Identification and nucleotide sequence of a gene in feline herpesvirus type-1 homologous to the herpes simplex virus gene encoding the glycoprotein B. *Archives of Virology* **127**, 387–397.

MAEDA, K., KAWAGUCHI, Y., ONO, M., INOSHIMA, Y., MIYAZAWA, T., TOHYA, Y., KAI, C. & MIKAMI, T. (1994). A gD homologous gene of feline herpesvirus type 1 encodes a hemagglutinin (gp 60). *Virology* **202**, 1034–1038.

OSTERHAUS, A. D. M. E. & VEDDER, E. J. (1988). Seal death. *Nature* **334**, 301–302.

OSTERHAUS, A. D. M. E., YANG, H., SPIJKERS, H. E. M., GROEN, J., TEPPEMA, J. S. & VAN STEENIS, G. (1985). The isolation and partial characterization of a highly pathogenic herpesvirus from the harbour seal (*Phoca vitulina*). *Archives of Virology* **86**, 239–251.

ROIZMAN, B., DESROSIERS, R. C., FLECKENSTEIN, B., LOPEZ, C., MINSON, A. C. & STUDDERT, M. J. (1992). The family *herpesviridae*: an update. *Archives of Virology* **123**, 425–436.

SPATZ, S. J. & MAES, R. K. (1993). Immunological characterization of the feline herpesvirus-1 glycoprotein B and analysis of its deduced amino acid sequence. *Virology* **197**, 125–136.

SPATZ, S. J., ROTA, P. A. & MAES, R. K. (1994). Identification of the feline herpesvirus type 1 (FHV-1) genes encoding glycoproteins G, D, I and E: expression of the FHV-1 glycoprotein D in vaccinia and racoon poxviruses. *Journal of General Virology* **75**, 1235–1244.

STENVERS, O., PLÖTZ, J. & LUDWIG, H. (1992). Antarctic seals carry antibodies against seal herpesvirus. *Archives of Virology* **123**, 421–424.

VEDDER, L., ZARNKE, R., SPIJKERS, I. & OSTERHAUS, A. D. M. E. (1987). Prevalence of virus neutralizing antibodies to seal herpesvirus (phocid herpesvirus 1) in different pinniped species. *Abstracts of the 7th Biennial Conference on Biology of Marine Mammals*, 5–9 December 1987, Miami, USA.

WHALLEY, J. M., ROBERTSON, G., BELL, C. W., LOVE, D., ELPHINSTONE, M., WILEY, L. & CRAVEN, D. (1991). Identification and comparative sequence analysis of a gene in equine herpesvirus 1 with homology to the herpes simplex virus glycoprotein D gene. *Virus Genes* **5**, 313–325.

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