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A novel porcine gammaherpesvirus

Barbara Chmielewicz,[a] Michael Goltz,[a] Tatjana Franz,[a] Cindy Bauer,[a] Susanne Brema,[a] Heinz Ellerbrok,[a] Sabine Beckmann,[a] Hanns-Joachim Rziha,[b] Karl-Heinz Lahrmann,[c] Carlos Romero,[d] and Bernhard Ehlers[a,*]

[a] *Robert Koch-Institut, Nordufer 20, D-13353 Berlin, Germany*

[b] *Bundesforschungsanstalt für Viruskrankheiten der Tiere, Institut für Immunologie, Paul-Ehrlich-Strasse 28, D-72076 Tübingen, Germany*

[c] *Freie Universität Berlin, Klinik f. Klauentiere, Königsweg 65, D-14163 Berlin, Germany*

[d] *College of Veterinary Medicine, 2015SW 16th Ave., Bldg. 1017, P.O. Box 110880, Gainesville, FL 32611, USA*

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Abstract

A novel porcine gammaherpesvirus was detected in the blood of domestic pigs by PCR. With degenerate-primer PCR and subsequent long-distance PCR approaches a 60-kbp genome stretch was amplified. Sequence analysis revealed the presence of the gammaherpesvirus ORFs 03 to 46 as well as a putative chemokine receptor and a v-bcl-2 gene. The 60-kbp sequence was compared with the corresponding sequence of the porcine lymphotropic herpesvirus 1 (PLHV-1) published recently and the sequence of PLHV-2, which was amplified from porcine tonsil. Considerable sequence differences (amino acid identities: 49–89%) were found between the novel virus and PLHV-1 as well as PLHV-2, which were very closely related to each other (amino acid identities: 85–98%). The novel virus had essentially the same genome organization as PLHV-1 and -2 and was therefore designated PLHV-3. Like PLHV-1 and -2, PLHV-3 was frequently found in the blood and in lymphoid organs of domestic and feral pigs from different geographic locations. In the blood, the PLHVs were detected predominantly in B-cells. Indication for latent as well as productive PLHV-3 infection was found in the porcine B-cell line L23. It can be concluded that the PLHVs are widespread and are likely to cause a persistent B-lymphotropic infection. Since PLHV-1 has been implicated in the development of porcine posttransplantation lymphoproliferative disease, all porcine lymphotropic gammaherpesviruses are of concern when pigs are used as donors in xenotransplantation.
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Introduction

In xenotransplantation, the absence of potentially pathogenic viruses in donor animals is an important safety issue. Since pigs are currently favored as donors of xenografts (Auchincloss and Sachs, 1998; O'Connell et al., 2000), attempts were undertaken to define the porcine viruses of concern and to breed pigs free of these viruses. Previously unknown porcine viruses were identified and a characterization of their biological properties was initiated (Iverson and Talbot, 1998; Meng et al., 1998; Onions et al., 2000; Patience et al., 1997).

We recently reported the discovery of DNA sequences of two closely related gammaherpesviruses in pigs—the porcine lymphotropic herpesviruses 1 and 2 (PLHV-1 and -2). These viruses are widespread in domestic pigs and are closely related to several ruminant gammaherpesviruses, most of which are etiologically implicated in the occurrence of malignant catarrhal fever (MCF), a lymphoproliferative and inflammatory disease with an often fatal outcome. PLHV-1 and -2 are also related to EBV, HHV-8, and other gammaherpesviruses, but more distantly (Ehlers et al., 1999b; Ulrich et al., 1999).

In allotransplantation, EBV is etiologically involved in a posttransplantation lymphoproliferative disease (PTLD) which represents a spectrum of lymphoid proliferations ranging from a reactive polymorphic expansion of EBV-

* Corresponding author. Fax: +49-1888-754-2598.
E-mail address: ehlersb@rki.de (B. Ehlers).

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infected lymphocytes to monoclonal B-cell lymphomas (Ferry and Harris, 1994). Evidence for a similar pathogenic potential of PLHV-1 was reported by Huang et al. (2001). Immunosuppressed miniature swine were subjected to allogeneic hematopoietic stem cell transplantation. Under these conditions, a high incidence of a syndrome very similar to human PTLD was observed, with most of the diseased animals dying. In the blood and lymph nodes, high amounts of a porcine gammaherpesvirus were detected by PCR. This was later found to be identical to PLHV-1. Furthermore, early and late genes of PLHV-1 were found to be transcribed in PTLD-affected pigs (Goltz et al., 2002). Thus, safe xenotransplantation with organs of swine requires the generation of pigs, which are both free of these and any other related porcine herpesviruses in order to minimize virus-related risks for transplanted patients.

The aim of the present study was therefore to search for unknown herpesviruses in porcine blood and tissues. A pan-herpes consensus PCR assay was used which targets a highly conserved region of the herpesviral DNA polymerase (DPOL) gene (Ehlers et al., 1999a). This approach had already enabled the discovery of PLHV-1 and PLHV-2 and other vertebrate herpesviruses (Chmielewicz et al., 2001; Ehlers et al., 1999a; 2001). Application of this assay to porcine blood and tissue samples resulted in the detection of a novel DPOL sequence indicating the presence of a previously unknown gammaherpesvirus. Here we present the characterization of a 60-kbp sequence of this novel virus and compare it with the corresponding sequences of PLHV-1 and PLHV-2, the latter being also determined in this study. Furthermore, we report on the analysis of PLHV prevalences in pigs, demonstrate the presence of the PLHVs in peripheral B-lymphocytes, and present evidence for PLHV-3 infection in a permanent porcine B-cell line.

Results

Detection of a novel porcine herpesvirus with pan-herpes PCR

A pan-herpes PCR assay targeting conserved regions of the herpesvirus DPOL gene was used to search for unknown gammaherpesvirus species in pigs. Since PLHV-1 and PLHV-2 have a high prevalence in domestic and feral pigs (Ehlers et al., 1999ab; Ulrich et al., 1999), we only screened blood and tissue samples free of PLHV-1 and PLHV-2 sequences. In the blood of a German domestic pig (sample no. 1412) a novel gammaherpesvirus-like DPOL sequence was detected which exhibited only 66% identity to the corresponding DPOL sequences of PLHV-1 and PLHV-2 and less than 60% to all other known gammaherpesviruses. The putative virus, from which the novel sequence originated, was tentatively designated "porcine lymphotropic herpesvirus 3" (PLHV-3). A search for other PLHV-3-positive samples using a specific PCR revealed a spleen sample (no. 489) which contained a higher copy number than the blood sample no. 1412. This was therefore used for determining the sequence of a genome stretch of PLHV-3.

Amplification and sequence analysis of 60 kbp of the PLHV-3 genome

A PCR-based approach was chosen because neither a lytical cell culture system nor a permanent cell line with sufficient copies of PLHV-3 was available at the beginning of this study. Initially, pan-gammaherpesvirus PCR assays were used to generate partial sequences of conserved genes (ORFs 03, 06, 08, 17, 25, 39, 44, and 46). The common block organization of the conserved gammaherpesvirus genes (for an overview, see Schulz, 1998; Simas and Efstathiou, 1998) enabled us then to amplify the interspersed sequences with PLHV-3-specific primers and long-distance PCR. Nine overlapping fragments of 3-12 kbp length were generated (Fig. 1a) and sequenced by primer walking. The 5′-part of ORF 03 was determined by genome walking (see Methods). A final 60326-bp sequence was generated, extending from the 5′-noncoding region of ORF 03 into ORF 46. It was verified with 616 overlapping PCR fragments resulting in an average redundancy of 5. The sequence has a G+C content of 39% and reveals a strong suppression of the CpG dinucleotide frequency.

Gene arrangement of PLHV-3

The PLHV-3 sequence determined includes the first and a major part of the second conserved gene block of the gammaherpesvirus subfamily. Upstream of the first gene block, an ORF coding for a viral phosphoribosylformylglycineamide amidotransferase (FGARAT; ORF 03) was found. Within the gene block, the major DNA-binding protein (ORF 06), a transport protein (ORF 07), the glycoprotein B (gB; ORF 08), the DNA polymerase (ORF 09), and two conserved ORFs with unknown function (ORFs 10 and 11) were identified. Interspersed between these were two ORFs which were not conserved among the gammaherpesviruses, E4/BALF1$_h$ and A5/BILF1$_h$ (Fig. 1a; Table 1). The ORF E4/BALF1$_h$ is homologous to genes which are at the same genomic position in EHV-2 (ORF E4), EBV (ORF BALF1), and two monkey lymphocryptoviruses, CeHV-15 (ORF BALF1) and CalHV-3 (ORF 01). BALF1 was reported to be a viral homolog of the mammalian bcl-2 oncogene (v-bcl-2), which encodes a functional apoptosis inhibitor (Marshall et al., 1999). The ORF A5/BILF1$_h$ is related to the ORFs A5 of A1HV-1, E6 of EHV-2, BILF1 of EBV, and CeHV-15 as well as ORF 06 of CalHV-3. No homologs are present in other rhadinoviruses. The encoded proteins have seven hydrophobic domains and may represent herpesviral G-protein-coupled receptors (McGeoch, 2001; Goltz et al., 2002). Within the second conserved gene block, genes encoding the thymidine kinase (TK, ORF 21), the glycoprotein H (gH, ORF 22), the major capsid protein

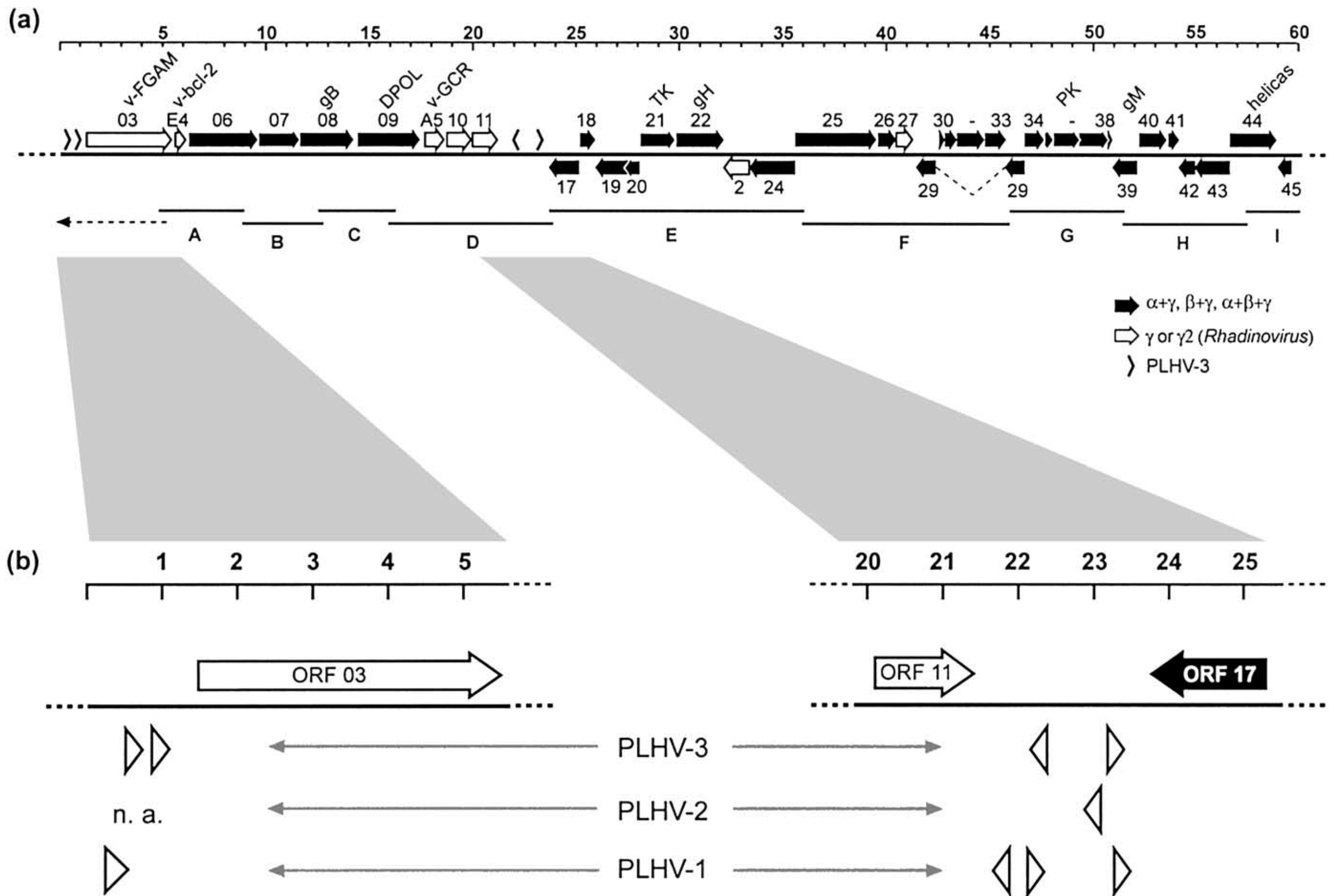

Fig. 1. Map of the open reading frames of the 60-kbp sequence of PLHV-3. (a) ORFs are shown as arrows. Description of ORFs and abbreviations are given in Table 1 (E4 = E4/BALF1$_h$; A5 = A5/BILF1$_h$). Black arrows indicate ORFs found in two or all three subfamilies of the herpesviruses. Open arrows symbolize ORFs described only for gammaherpesviruses. Black lines, designated A to I, symbolize the amplimers generated with long-distance PCR which demonstrate the contiguity of the PLHV-3 sequence. The dashed arrow symbolizes a PCR amplimer generated by genome walking. A scale in kbp is given above. Base 1 is the first base of the genome stretch determined in this study. This position is not identical with the beginning of the long unique region of PLHV-3, which has not yet been determined. (b) The two genome regions coding for ORFs, which are not conserved among the PLHVs and are not found in other herpesviruses, are shown enlarged. Scale and symbols for conserved ORFs refer to part (a) of the figure. The position and orientation of the non-conserved ORFs are symbolized by gray arrows. For PLHV-2, sequence information upstream of ORF 03 is not available (n.a.), as indicated.

(MCP, ORF 25), the terminase (ORFs 29a,b), the glycoprotein M (gM, ORF 39), and the helicase (ORF 44) as well as an immediate early gene (ORF 45) and part of the uracil-DNA-glycosidase gene (ORF 46) were identified (Fig. 1a; Table 1).

Upstream of ORF 03 and between the ORFs 11 and 17, four short ORFs were found which did not match significantly in database searches and did not reveal counterparts in PLHV-1 and PLHV-2 (Fig. 1b).

PLHV-3 is a distant member of the PLHV group

The 60-kbp sequence of PLHV-3 was compared with the corresponding sequences of PLHV-1, PLHV-2, and other gammaherpesviruses. For this purpose, the 60-kbp sequence of PLHV-2 also had to be determined, since only 4.1 kbp had been sequenced previously (Ulrich et al., 1999). This was done by amplification from DNA of a PLHV-2-positive, PLHV-1- and PLHV-3-negative tonsil sample (no. 568) of a German feral pig as described under Methods. A 73-kbp PLHV-1 sequence had been determined earlier from DNA of a Spanish domestic pig (Goltz et al., 2002). In Table 1, the potential PLHV-3 genes and their homologs in PLHV-1, PLHV-2, A1HV-1 (the most closely related non-porcine rhadinovirus), and HHV-8 and in the lymphocryptovirus EBV are listed.

The gene organization of PLHV-3 is identical to that of PLHV-1 and -2 in the analyzed region of the genome. This applies not only to the conserved herpesvirus ORFs but also to the gammaherpesvirus-specific ORFs 03, 10, 11, 23, and 27, as well as for the nonconserved ORFs E4/BALF1$_h$ and A5/BILF1$_h$ (Table 1). However, the amino acid identity values of 49–89% between PLHV-3 and PLHV-1 or PLHV-2 clearly show that PLHV-3 is far more distantly related to PLHV-1 and PLHV-2 than these two viruses are to each other (85–98%; Table 1). The amino acid identity

Table 1
Potential PLHV-3 ORFs and homologs to PLHV-1, PLHV-2, AIHV-1, HHV-8, and EBV

PLHV		PLHV-3 Size (aa)	PLHV-1		PLHV-2		PHLV-1/PLHV-2	AIHV-1		HHV-8		EBV			Description
ORF	Pol		%id	size	%id	size	%id	%id	size	%id	size	%id	size	ORF	
P1.1	+	—	—	107		n.a.		—	—	—	—	—	—	—	
P3.1	+	73	—	—		n.a.		—	—	—	—	—	—	—	
P3.2	+	97	—	—		n.a.		—	—	—	—	—	—	—	
03[a]	+	1378	69.5	1378	69.7	1376	90.6	32.5	1369	—	—	—	—	—	Phosphoribosylformylglycineamide amidotransferase (v-FGARAT)
E4/BALF1	+	219	81.9	178	82.6	178	92.1	—	—	—	—	22.3	220	BALF1	v-bcl-2
06	+	1125	80.5	1126	80.2	1142	95.9	56.9	1127	48.7	1133	44.0	1128	BALF2	Major DNA-binding protein (MDBP)
07	+	674	73.8	675	73.1	674	91.2	50.0	680	43.0	695	38.2	789	BALF3	Transport protein
08	+	876	77.5	876	78.1	876	93.4	56.8	854	45.7	845	44.9	857	BALF4	Glycoprotein B (gB)
09	+	1001	80.1	1004	79.7	1001	95.1	63.1	1026	57.6	1012	53.2	1015	BALF5	DNA polymerase (DPOL)
A5/BILF1	+	338	61.3	325	63.7	304	89.5	31.1	302	—	—	22.0	312	BILF1	G-protein-coupled receptor (v-GCR)
10	+	400	59.6	401	60.6	417	90.3	26.6	404	20.7	418	[b]	422	RAJILF1	
11	+	410	63.8	409	63.4	409	89.5	41.8	406	26.2	408	31.8	429	RAJILF2	
P1.2	−	—	—	77	—	—	—	—	—	—	—	—	—	—	
P3.3	−	71	—	—	—	—	—	—	—	—	—	—	—	—	
P1.3	+	—	—	78	—	—	—	—	—	—	—	—	—	—	
P2.1	−	—	—	—	—	80	—	—	—	—	—	—	—	—	
P1.4	+	—	—	69	—	—	—	—	—	—	—	—	—	—	
P3.4	+	68	—	—	—	—	—	—	—	—	—	—	—	—	
17	−	527	66.1	500	65.2	500	91.4	45.1	524	43.2	553	35.3	605	BVRF2	Capsid protein
18	+	258	72.6	261	74.5	261	91.6	44.9	275	41.5	257	—	—	—	
19	−	555	71.7	549	71.0	549	91.6	50.7	556	41.4	549	36.5	570	BVRF1	Tegument protein
20	−	254	71.8	275	73.8	232	93.1	39.5	250	42.2	320	32.4	248	BXRF1	
21	+	574	71.7	580	71.3	578	86.7	36.1	561	30.6	580	37.5	607	BXLF1	Thymidin kinase (TK)
22	+	772	68.9	778	69.5	778	90.6	36.6	733	27.0	730	28.5	706	BXLF2	Glycoprotein H (gH)
23	−	412	75.5	398	73.4	399	91.5	43.9	401	31.3	404	33.3	425	BTRF1	
24	−	734	73.3	736	72.5	736	91.2	51.3	745	42.4	752	41.8	575	BcRF1	
25	+	1376	88.5	1372	88.3	1372	97.7	67.3	1370	57.4	1376	55.1	1381	BcLF1	Major capsid protein (MCP)
26	+	304	82.9	304	83.3	304	97.7	60.6	306	47.5	305	49.2	301	BDLF1	Capsid protein
27	+	310	69.1	294	70.2	294	90.5	41.5	292	29.0	290	20.7	420	BDLF2	
29	−	684	80.5	683	81.0	683	96.0	62.1	686	54.4	687	51.6	690	BDRF1	Cleavage/packaging protein
30	+	80	49.4	79	50.6	91	84.8	30.0	85	30.0	78	21.9	77	BDLF3.5	
31	+	214	72.8	206	73.4	206	95.6	41.6	225	38.6	224	36.7	225	BDLF4	
32	+	455	62.7	453	60.4	453	89.8	35.9	474	27.4	454	22.2	507	BGLF1	DNA cleavage/packaging protein
33	+	339	85.8	339	85.5	347	96.2	45.4	335	32.7	312	45.6	336	BGLF2	
34	+	324	73.1	326	73.5	326	93.9	46.8	343	37.1	327	34.2	332	BGLF3	
35	+	146	61.6	151	61.9	151	93.4	34.0	152	26.0	150	26.2	153	BGLF3.5	
36	+	454	75.1	453	74.2	453	95.6	42.8	454	27.3	444	29.0	455	BGLF4	Kinase (PK)
37	+	485	87.6	485	91.1	485	97.6	62.1	485	42.9	486	45.7	470	BGLF5	Alkaline exonuclease
38	+	61	80.6	63	83.9	63	92.1	41.4	59	27.1	61	37.1	75	BBLF1	
39	−	382	79.1	378	78.4	378	93.4	65.2	374	44.9	400	49.9	405	BBRF3	Glycoprotein M (gM)
40	+	449	52.8	455	51.7	467	85.9	30.8	478	26.8	457	23.6	522	BBLF2	Helicase-primase complex component
41	+	180	54.9	162	55.8	162	89.5	24.9	175	25.9	205	35.3	201	BBLF3	Helicase-primase complex component
42	−	267	78.2	266	79.0	266	89.9	44.9	257	36.0	278	37.5	278	BBRF2	
43	−	567	80.6	567	80.7	566	95.2	62.7	557	54.9	605	48.8	613	BBRF1	Capsid protein
44	+	843	82.1	782	82.2	782	97.3	63.4	783	55.6	788	50.6	809	BBLF4	Helicase
45	−	178	63.3	223	60.4	223	89.7	38.3	235	30.1	407	27.0	217	BKRF4	

Pol, polarity; ORF, orientation; %id, percentage amino acid identity to the corresponding PLHV-3 ORF; n.a., not available; for PLHV-2 no sequence information upstream of ORF 03 is available.

[a] The nomenclature of the ORFs 03 to 45 is identical in PLHV-1, PLHV-2, PLHV-3, AIHV-1, and HHV-8.

[b] Identity value below 20%.

values between PLHV-3 and other gammaherpesviruses are less than 67%, as exemplified by comparison with A1HV-1, HHV-8 and EBV (Table 1).

Phylogenetic analysis was based on amino acid sequence alignments which were performed with a set of conserved genes (ORFs 06, 08, 09, 29, and 44). The five alignments were concatenated, regions with gaps were removed and the resulting composite alignment (3788 aa) was analyzed with the neighbor/joining method. The phylogenetic tree showed branching of PLHV-3 with the PLHV-1/PLHV-2 pair and, more distantly, with AIHV-1. This was confirmed with parsimonial analysis (program Protpars) and with analyses

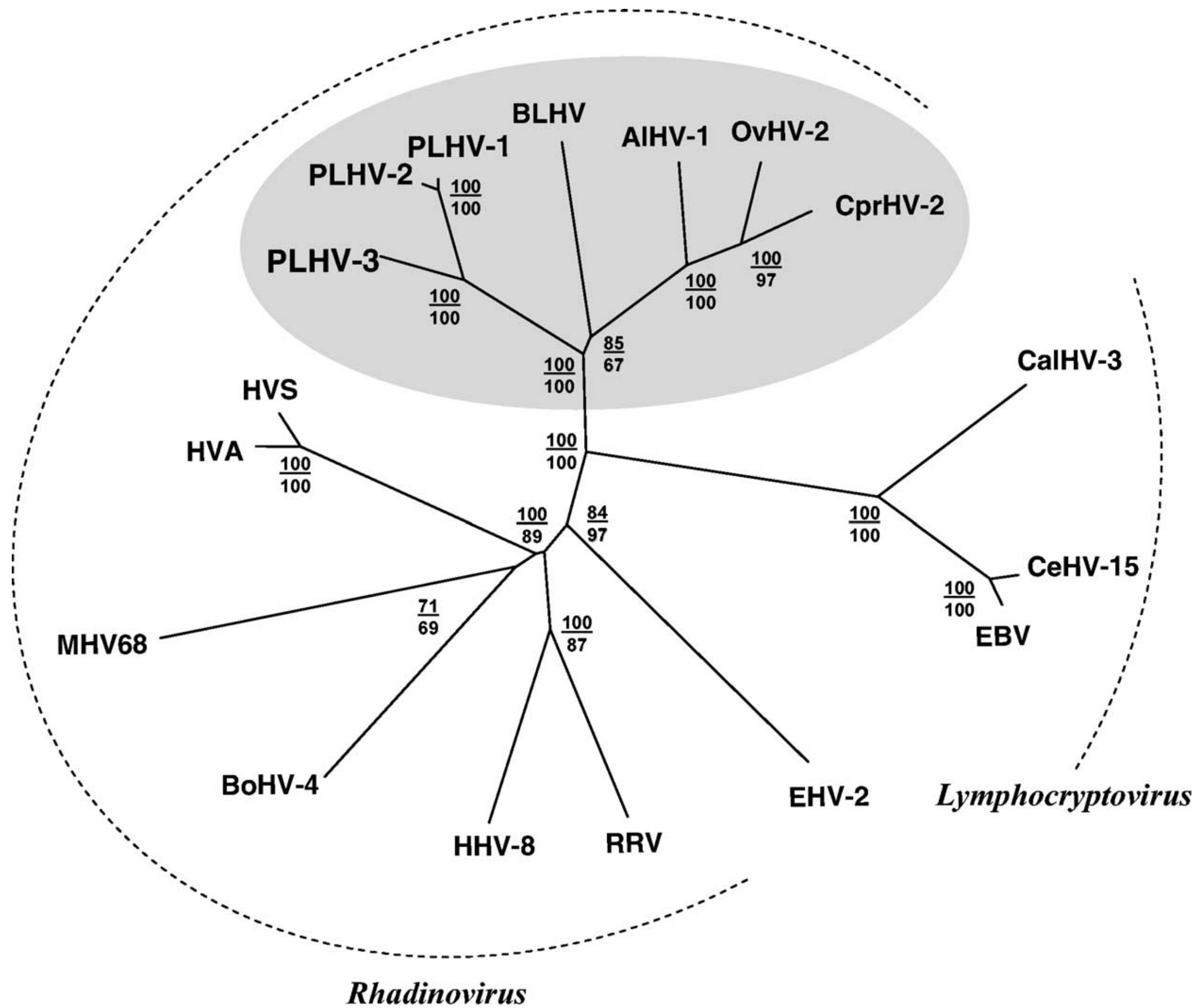

Fig. 2. Phylogenetic relatedness of PLHV-3. A phylogenetic tree was constructed with the neighbor/joining method, using concatenated multiple amino acid sequence alignments of conserved regions of gB and DPOL. The PLHV-3 and PLHV-2 amino acid sequences were deduced from the nucleotide sequences determined in this study. Amino acid sequences of other gammaherpesviruses were obtained from GenBank (Accession Nos. under Methods). The bootstrap values derived from 100 repetitions are indicated at the branching points of the tree. The upper values were obtained with neighbor/joining analysis, the lower values with parsimonial analysis. A low bootstrap value was obtained at one node in the center of the tree and therefore not shown. Viruses classified to the genus *Rhadinovirus* and to the genus *Lymphocryptovirus* are indicated as such. The PLHVs and several ruminant gammaherpesviruses form a separate clade which is highlighted by gray background.

of individual proteins with both algorithms (data not shown). Phylogenetic analysis of DPOL and gB sequences (684 aa), in which ruminant gammaherpesviruses could be additionally included, placed the PLHVs similarly close to AIHV-1, OvHV-2, BLHV, and CprHV-2. Collectively, these seven viruses of artiodactyla form a separate clade within the gammaherpesvirus tree (Fig. 2).

The PLHVs are widely distributed and genetically stable

To determine the prevalence of PLHV-3 in different herds of commercial domestic pigs in Germany, PCR with the primer pairs 886-S/886-AS (DPOL) and 905-S/905-AS (gB) (Table 2) was performed. Peripheral blood leukocyte (PBL) samples ($n = 92$) from 16 different herds (5–9 pigs/herd) were tested, and 47 samples (51%) originating from 14 herds were positive for PLHV-3 (Table 3). More than 90% of the positive samples reacted in both PCR assays which indicated that both assays had a very similar sensitivity. For some of the amplimers the PLHV-3 origin was controlled by sequence analysis.

Real-time PCR assays specific for PLHV-1, PLHV-2, and PLHV-3 were also established (Table 2) and applied to the same collection of blood samples, since the detection limits had been determined to be less than 20 copies of viral genome. The results for PLHV-3 (48%) were in agreement with the data obtained by conventional PCR (51%). PLHV-2 was found in 16% and PLHV-1 in 54% of the samples (Table 3). The prevalence in lung ($n = 27$) and spleen ($n = 34$) from German pigs was also tested with

Table 2
Primers

Primer name	Gene	Primer sequence	T_{ann} (°C)[a]
PLHV-3-specific primers for prevalence analyses			
905s	ORF08 (gB)	5′-ACA AGA GCC TTA GGG TTC CAA ACT-3′	57
905as	ORF08 (gB)	5′-GTG TCC AGT GTT GTA ATG GAT GCC-3′	
886s	ORF09 (DPOL)	5′-CAA GAT TGC TGA GAC GGT GAC TAC-3′	57
886as	ORF09 (DPOL)	5′-AAA TGG CAT GGT TAC ATC TTT AGG-3′	
TaqMan primers and probes			
1125s	PLHV-1 gB	5′-CTC ACC TCC AAA TAC AGC GA-3′	55
1125as		5′-GCT TGA ATC GTG TGT TCC ATA G-3′	
Probe		5′-FAM-CTG GTC TAC TGA ATC GCC GCT AAC AG-TAMRA-3′	
1155s	PLHV-2 gB	5′-GTC ACC TGC AAA TAC ACA GG-3′	55
1155as		5′-GGC TTG AAT CGT ATG TTC CAT AT-3′	
Probe		5′-FAM-CTG GTC TAC TGA AGC GCT GCC AAT AG-TAMRA-3′	
1156s	PLHV-3 gB	5′-AAG GAC CCC AAA GAG GAA A-3′	55
1156as		5′-CTG AGG CAC TGC ATA CTC TGT-3′	
Probe		5′-FAM-TCA ATT TTA TGG TTC ACC TTC TAC CTT TCC T-TAMRA-3′	
468s	β-Actin	5′-TCA CCC ACA CGG TGC CCA TCT ACG A-3′	67
468as		5′-CAC CGG AAG CGC TCG TTG CCG ATG G-3′	
Probe		5′-FAM-ACG CCC TGC CCC ACG CCA TCC TGC GT-TAMRA-3′	

[a] T_{ann}, annealing temperature; the values apply for the combination of the respective sense primer with the antisense primer below.

real-time PCR. PLHV-1, -2, and -3 were found in 78, 41, and 59% of the lung samples and in 59, 26, and 62% of the spleen samples, respectively. In blood samples from 20 Italian domestic pigs, prevalences of 80, 20, and 65% were found for PLHV-1, -2, and -3 respectively (Table 3). Of all 173 samples analyzed, 5 were positive for PLHV-1 and PLHV-2, 46 for PLHV-1 and PLHV-3, and 11 for PLHV-2 and PLHV-3; 17 were positive for all three PLHVs. None of the PLHVs were found in 23 samples.

PCR analysis of additional samples from domestic and feral pigs from Germany, France, Spain, and the USA revealed the presence of the three PLHV species in each of these countries. For each PLHV species, six samples from the different sources were investigated by sequence analysis of 0.5 kbp of the gB gene to detect possible intraspecies sequence differences. The gB region was chosen because it is variable in different strains of some herpesvirus species (Chou and Dennison, 1991; Lehner et al., 1991; Searles et

Table 3
Prevalence of the PLHV viruses

Tissue	Farm	Country	N	DPOL PCR PLHV-3	gB PCR PLHV-3	gB real-time-PCR		
						PLHV-3	PLHV-2	PLHV-1
Blood	1	Ger	5	2	2	2	2	4
Blood	2	Ger	6	2	2	2	0	6
Blood	3	Ger	6	3	3	3	1	1
Blood	4	Ger	5	4	5	5	1	4
Blood	5	Ger	7	7	6	6	1	4
Blood	6	Ger	6	2	3	2	0	5
Blood	7	Ger	5	3	3	3	0	3
Blood	8	Ger	5	5	4	4	2	5
Blood	9	Ger	5	1	1	0	0	3
Blood	10	Ger	5	5	5	5	3	2
Blood	11	Ger	5	2	2	2	1	3
Blood	12	Ger	5	3	3	3	0	1
Blood	13	Ger	5	4	3	4	1	3
Blood	14	Ger	8	4	5	3	2	0
Blood	15	Ger	5	0	0	0	1	0
Blood	16	Ger	9	0	0	0	0	6
		Σ	92	47 (51%)	47 (51%)	44 (48%)	15 (16%)	50 (54%)
Blood		It	20	n.d.	n.d.	13 (65%)	4 (20%)	16 (80%)
Lung		Ger	27	n.d.	n.d.	16 (59%)	11 (41%)	21 (78%)
Spleen		Ger	34	n.d.	n.d.	21 (62%)	9 (26%)	20 (59%)

Ger, Germany; I, Italy; n.d., not done; Σ, sum.

al., 1999). In addition, 0.4 kbp of the weakly conserved intergenic sequences between ORFs 11 and 17 were amplified and sequenced. The variations found within each PLHV species were below 0.1% for the gB gene and below 1% for the intergenic region. A 25-kbp sequence of PLHV-3 (ORFs 03 to 17) was also determined from blood sample no. 1412. This sequence and the corresponding region of the 60 kbp PLHV-3 sequence (spleen sample no. 489) differ by less than 0.1%. Finally, the 60-kbp sequence of PLHV-2 (tonsil sample no. 568) was compared to a second PLHV-2 sequence of 32 kbp length (tonsil sample no. 546). Differences below 0.1% were observed (not shown). These data indicate that the PLHVs are genetically stable, irrespective of their origin.

Presence of the PLHVs in PBMC subpopulations

To determine the cell types infected by the PLHVs in porcine peripheral blood mononuclear cells, PBMCs were isolated from 12 PLHV-positive pigs. They were incubated with monoclonal antibodies specific for porcine B-, T-, or myeloid cells (anti-immunoglobulin, anti-CD3, and anti-SWC3, respectively), followed by an incubation with anti mouse IgG-Microbeads and purification of cells over a MACS column. The resulting cell populations were analyzed with real-time PCR for detection of PLHV-1, -2, or -3. Nine out of 12 animals tested positive for PLHV-3, 2 for PLHV-2, and 9 for PLHV-1 in the unsorted PBMC samples (Fig. 3). The copy numbers of viral DNA in the unfractionated PBMC were 1×10^0–8×10^4 (PLHV-3), 1.8×10^1–$4{,}1\times10^1$ (PLHV-2), and 1×10^0–2.8×10^2 (PLHV-1) per 10^4 cells. Analysis of the subpopulations showed a higher or at least similar copy number of viral DNA in B-cells, compared with the unfractionated PBMCs from 8 of 9 PLHV-3-infected, 4 of 9 PLHV-1-infected, and both PLHV-2-infected animals. Copy numbers which were comparable to those in the unsorted cells were also found in the myeloid fractions from three pigs (one PLHV-3 and two PLHV-1). Furthermore, the myeloid fraction from one of the pigs, which tested positive for PLHV-1, was the only population in which viral DNA was detectable. The T-cell fractions were either completely negative or had only relatively low copy numbers of viral DNA (Fig. 3). This detection of viral DNA may be attributed to contamination of the T-cell fraction with virus-carrying non-T-cells, although this cell population always had a very high purity (98.9–99.8%). In 4 of 9 PLHV-1-positive pigs all three cell fractions contained much lower PLHV-1 copy numbers than the unfractionated PBMCs. The sum of the copy numbers of the three subpopulations was also much lower than that of the unsorted PBMCs of these pigs. This indicates that a major part of the virus-carrying cells was not targeted by the applied sorting conditions.

Identification of PLHV-3 in a permanent porcine B-cell line

More than 20 permanent porcine cell lines—including one of B-cell origin—were tested for the presence of PLHV

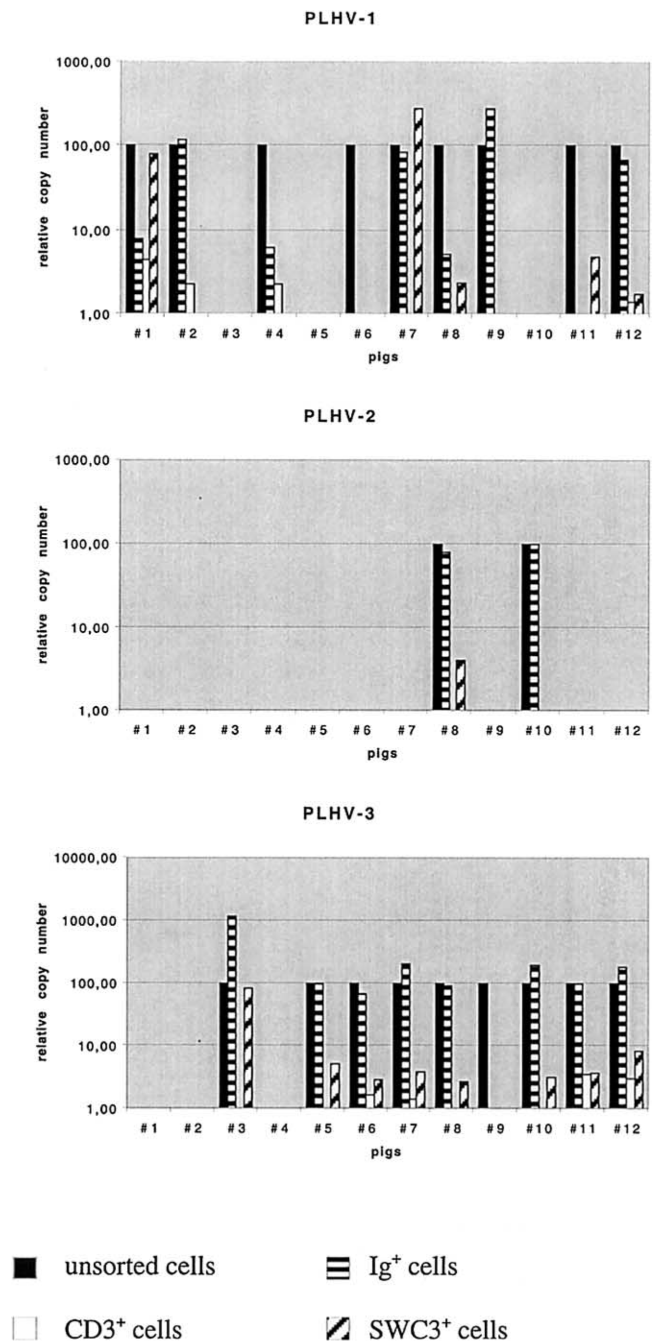

Fig. 3. Presence of the PLHVs in PBMC subpopulations. B-cells, T-cells, and myeloid cells were purified and tested with real-time PCR for the presence of PLHV-1, PLHV-2, or PLHV-3 as described under Methods. The relative DNA copy numbers of PLHV-1, -2, and -3 are shown for the unsorted cells, the Ig^+ cells, the $CD3^+$ cells, and the $SWC3^+$ cells of 12 pigs. In the unsorted cell samples the absolute copy numbers of viral DNA were normalized to 100 and the copy numbers in the B-, T-, and myeloid cell fractions calculated accordingly.

DNA with PCR. All cell lines were negative, with one exception. The B-cell line L23 was found to contain the PLHV-3 genome when tested with 14 PCRs of 3–6 kbp length covering the complete 60-kbp sequence of PLHV-3 (not shown). With real-time PCR, approximately 400

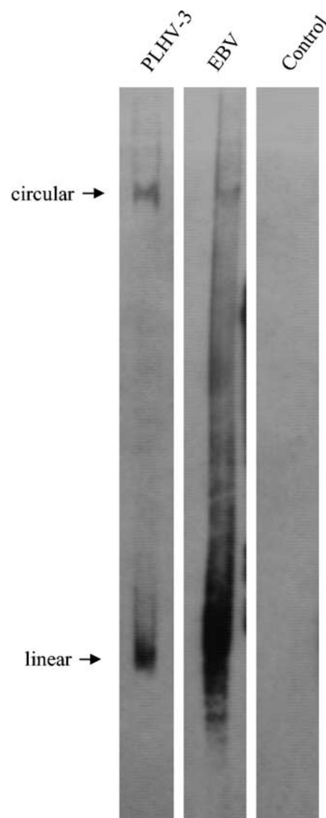

Fig. 4. Gardella gel analysis of PLHV-3 in the porcine B-cell line L23. PLHV-3-infected L23 cells (3×10^6) (Lane 1), 1×10^6 EBV-infected B95-8 cells for positive control (Lane 2), and 5×10^6 Molt 4 cells for negative control (Lane 3) were subjected to Gardella gel electrophoresis, blotted, and hybridized to a mixture of all DIG-labeled probes as described under Methods. Indicated by arrows are bands which represent circular and linear viral genomes. The three parts of the figure originate from the same blot and are a representative example of several experiments.

PLHV-3 genome copies per cell were determined. Gardella gel electrophoresis was performed to demonstrate genomes complete PLHV-3 genomes in the L23 cell line. The Gardella gel was blotted and hybridized with a mixture of PLHV-3-specific probes. As a positive control, the EBV-positive B95-8 cell line was hybridized with two EBV-specific probes. The $CD4^+$ human cell line Molt 4, which does not carry any herpesviruses, was used as a negative control. As shown in Fig. 4, two hybridization signals were seen in the B95-8 cell line. The more slowly migrating band originates from episomal circular EBV genomes that are associated with latent infection, while the fast migrating band originated from linear EBV genomes packaged into virus particles, as reported earlier (Gardella et al., 1984). Analysis of the L23 cell line revealed one band migrating as slowly as the episomal circular EBV genomes and a second band migrating slightly faster than the linear EBV genomes. This was confirmed in several independent experiments. It indicated the presence of circular as well as linear PLHV-3 genomes in L23 cells.

Discussion

This study represents a firm identification of the third porcine gammaherpesvirus species—PLHV-3—and its initial characterization. One of our goals was to perform a comprehensive comparison of a sequence of the novel virus with that of both PLHV-1 and PLHV-2 and was based on genome stretches of 60 kbp. The results of amino acid sequence comparisons of 37 homologous genes and of phylogenetic analyses clearly indicate that PLHV-3 forms a group with PLHV-1 and PLHV-2 which is distinct from other gammaherpesviruses. However, PLHV-3 is far more distantly related to PLHV-1 and PLHV-2 than these two viruses are to each other (Table 1, Fig. 2).

PLHV-3 exhibits, like PLHV-1 and PLHV-2, a high intraspecies identity (>99%). This is in accordance with a previous analysis of the PLHV-1 and PLHV-2 DPOL loci (Ehlers 1999a,b; Ulrich 1999) and the PLHV-1 gB-locus (Goltz et al., 2002). It indicates that the PLHVs are genetically stable viruses which may be perfectly adapted to their natural host. It also ensures the conservation of the primer-binding sites used in the PCR and real-time PCR assays.

An important observation is that the gene content of the PLHVs is very similar to that of the wildebeest-associated MCF virus AlHV-1, as shown for PLHV-3 and PLHV-2 in this study and for PLHV-1 by Goltz et al. (2002). There is also a close relationship to the sheep-associated MCF virus OvHV-2, the bovine leukemia-associated BLHV, and the recently described goat gammaherpesvirus CprHV-2, which may be associated with a chronic disease syndrome of Sika deer (Fig. 2; Ehlers et al., 1999a; Chmielewicz et al., 2001; Li et al., 2001; Rovnak et al., 1998). Both AlHV-1 and OvHV-2 are well adapted to their natural hosts, since they are apathogenic in wildebeest and sheep. However, when they infect foreign hosts like cattle or deer, they cause MCF, a lymphoproliferative and inflammatory disease which is fatal in most cases (Metzler, 1991; Plowright et al., 1960). Recently, the potential of OvHV-2 to infect genetically distant hosts was demonstrated when pigs became OvHV-2 infected and developed fatal MCF through contact with OvHV-2-positive sheep (Loken et al., 1998; Albini, et al., 2003). Both this and the recently reported association of PLHV-1 with a lymphoproliferative disease in immunosuppressed, allotransplanted pigs (Goltz et al., 2002; Huang et al., 2001) raise the concern that the PLHVs might cause a similar lymphoproliferative syndrome in a pig-to-human xenotransplantation.

According to the amino acid sequence comparisons (Table 1) and phylogenetic analyses (Fig. 2), the partial genomes of the PLHVs have a gene content and arrangement very similar to the ruminant rhadinovirus AlHV-1. In addition, the low G+C content (<40%) and the strong suppression of the CpG dinucleotide frequency are indicative for rhadinoviruses (Ulrich et al., 1999). However, Goltz et al. (2002) presented several lines of evidence that PLHV-1 may be closer related to the lymphocryptovirus EBV than to

other nonruminant rhadinoviruses. This may favor a division of the genus *Rhadinovirus* into two genera. However, the complete PLHV genome sequences have to be determined to enable a final classification.

In this study, experimental evidence for a B-cell tropism of the PLHVs was obtained. The identification of PLHV-3 DNA in the permanent porcine B-cell line L23 demonstrated the infection of this cell type. Circular and linear genomes were detected by the Gardella technique (Fig. 4), indicating latent and productive viral infection. In vivo, with real-time PCR high PLHV DNA copy numbers were found predominantly in the B-cell fraction and less frequently in the myeloid fraction of PBMCs from 12 pigs. They were not found in the T-cell fraction (Fig. 3). Interestingly, although the B-cells appeared to be the main target cells for the PLHVs, in the majority of the samples the viral copy numbers were only slightly higher or even lower as compared to the unsorted control. This indicates that a major part of the virus-carrying cells was not targeted by the applied sorting conditions. One possible explanation might be the infection of a cell type which was not targeted by the antibodies used in this study. A possible candidate is the natural killer (NK) cell, which has a $CD3^{-}$ phenotype in pigs (Yang and Parkhouse, 1996) and was discussed as the proliferating cell type in MCF of cattle (Swa et al., 2001). Presuming a tropism mainly for B-cells, a second explanation could be the downregulation of IgM molecules on the cell surface caused by the virus infection of the cell. Such a downregulation was observed after expression of an HHV-8 protein (K1) in BJAB-cells, an HHV-8-negative B-cell line. This protein causes incorrect assembly of the IgM-containing B-cell antigen receptor complex, which leads to a retention of the complex in the endoplasmatic reticulum and therefore to a lowered expression of IgM at the cell surface (Lee et al., 2000). Such a lowered expression of the IgM molecule would lead to a less efficient enrichment of the virus-infected cells compared with the noninfected cells and result in the moderate copy numbers found in our experiments. However, it cannot be entirely ruled out that the binding characteristics of the anti-immunoglobulin antibody were the reason for the results obtained. The antibody was raised against an IgG molecule, but recognizes all Ig subclasses (as stated by the manufacturer). If this antibody had a different affinity to the Ig subclasses and the maturation stages of B cells also had different virus loads, then the obtained copy numbers would give incomplete information. However, this alone cannot fully explain the moderate levels of viral genome copies in the B-cells. In summary, from the presented data it can be concluded that the B-cell is a major target cell for PLHV infection in pigs. The presence of the PLHVs in most compartments of the body can thus be inferred and raises concern for the use of tissues and organs in pig-to-human xenotransplantation.

PCR and real-time PCR analyses revealed that more than 80% of domestic pigs are infected with one or more PLHV species (Table 3). This percentage even rose further to >90% when the samples included in this study were analyzed by PCR for the presence of the porcine cytomegalovirus (PCMV), a highly prevalent porcine betaherpesvirus (Goltz et al., 2000; B. Ehlers and M. Goltz, unpublished data). For several herpesviruses, including PCMV, intrauterine transmission has been reported (Edington et al., 1977). There is also preliminary data indicating that this is also the case for PLHV-1 (C. Patience, personal communication). Taking these considerations into account, elimination of herpesviruses from pigs by clean-catch procedures using cesarean section or medicated early weaning (Alexander et al., 1980) will be difficult. In addition, evaluation of the PLHV status of live pigs by analysis of their blood may give an underestimate as indicated by the higher prevalence values found in organs as compared to blood samples (Table 3). Although comparative PCR analyses of organ and blood samples from the same pigs are necessary to address this question more precisely, these data indicate that the development of reliable antibody-based assays for serological testing of pigs is mandatory. For this purpose, PLHV structural genes are being cloned and expressed.

The identification and initial biological characterization of the third porcine gammaherpesvirus species, PLHV-3, is a further important step in exploring the herpesvirus species infecting pigs for the benefit of safe xenotransplantation. From all data currently available it can be concluded that the three PLHV species represent B-cell tropic viruses with a lymphoproliferative potential. So far only PLHV-1 was shown experimentally to be associated with porcine lymphoproliferative disease. However, due to the close relationship of PLHV-2 and PLHV-3, it can be assumed that these viruses also cause lymphoproliferative disease under the same experimental conditions. Analysis of the complete PLHV genomes and development of a lytic cell culture system will help to further characterize the porcine lymphotropic herpesviruses and to assess their disease potentials in pig-to-human xenotransplantation.

Methods

Sample collection and DNA preparation

Blood and tissue samples were collected and DNA was prepared as described previously (Ulrich et al., 1999). Samples were also collected from experimental pigs housed at the Bundesforschungsanstalt für Viruskrankheiten der Tiere (BFAV) in Tübingen, Germany, the Klinik für Klauentiere at the Freie Universität Berlin, Germany, and from feral pigs hunted in Florida, USA. DNA samples from French, Italian, and Swedish domestic pigs were kindly provided by A. Jestin, Dario Di Luca, and Frederik Widen. DNA samples from miniature swine, kept at the Transplantation Biology Research Unit (TBRC), Massachusetts General Hospital, Harvard Medical School, Boston, USA, were kindly

provided by Chris Huang (TBRC) and Clive Patience (Immerge Biotherapeutics Inc., Boston, USA).

Amplification of PLHV-3 sequences with PCR

Pan-gammaherpesvirus PCR assays were carried out as single-round, semi-nested or nested PCR. Primers were degenerate and/or deoxyinosine substituted and published previously for ORF 08 (glycoprotein B gene) and ORF 09 (DNA polymerase gene) (Chmielewicz et al., 2001; Ehlers et al., 1999a). Degenerate primers were also designed for the gammaherpesvirus ORFs 03, 06, 17, 25, 39, 44, and 46 (primers not listed). PCR mixtures (25 μl) contained 1 μM of each primer. All other reaction conditions were as described earlier (Ehlers et al., 1999b).

PLHV-3 sequences extending upstream from ORF 03 were amplified by Genexpress GmbH (Berlin) using the Universal GenomeWalker kit from Clontech Laboratories, Inc., as described by Ulrich et al. (1999).

For specific amplification of PLHV-3, amplimers of 3–12 kbp length were generated with the Expand-Long-Template System (Roche Diagnostics, Germany) according to the manufacturer's instructions (primers not listed). To analyze the PLHV-3 prevalence, glycoprotein B and DPOL sequences were specifically amplified with the primer pairs 905s/905as (product size: 256 bp) and 886s/886as (product size: 148 bp), respectively, using AmpliTaq Gold (Perkin Elmer) as published (Ehlers et al., 1999a) (Table 2).

Amplification of PLHV-2 sequences with specific PCR

The high sequence similarity between PLHV-1 and PLHV-2 DPOL genes (Ulrich et al., 1999) enabled the use of primers, which had been already used for specific amplification of PLHV-1 DNA (Goltz et al., 2002), for amplification of PLHV-2 DNA. Approximately 75% of the region between ORF 03 and ORF 46 could be amplified. Remaining gaps were closed with primers deduced from the newly determined PLHV-2 sequences. The 5′-part of PLHV-2 ORF 03 was amplified as described above for ORF 03 of PLHV-3.

Cloning of glycoprotein B sequences

For standardization of real-time PCR assays, amplimers of partial glycoprotein B sequences of PLHV-1, -2, and -3 were cloned into the vector pCR 2.1 TOPO according to the manufacturer's instructions (TOPO TA cloning kit, Invitrogen).

Real-time PCR (TaqMan) for PLHV-1, PLHV-2, and PLHV-3

Real-time PCR was performed using primers and probes specific for the glycoprotein B gene of PLHV-1, PLHV-2, and PLHV-3 and the porcine β-actin gene respectively (Table 2). PCR contained 300 nM of both sense and antisense primers, except for the sense primer for PLHV-3 (900 nM) and the sense primer for β-actin (100 nM). All probes were used in a concentration of 100 nM. The reaction mixtures contained the TaqMan Universal PCR Master Mix (Applied Biosystems) at a 50% lower concentration as suggested by the manufacturer. The reaction was performed in an ABI PRISM 7700 Sequence Detection System (Perkin Elmer) as follows: 2 min at 50°C (AmpErase reaction) and 10 min at 95°C (denaturation) were followed by 45 cycles at 95°C for 20 s and annealing/extension at 55°C (PLHV-1, -2, -3) or 67°C (β-actin) for 1 min. All samples were tested in triplicate; from the three resulting C_t values the median was used for further calculations. Standard curves were generated using 10^1 to 10^6 copies of the plasmids containing the PLHV target sequences. For all three viruses the detection limit was determined to be 20 copies; 10 copies could be detected in 5 of 6 PCRs. Cross-reactivity of the PCR systems between the three PLH viruses as well as with porcine genomic DNA was also tested. No cross-reactions were observed, neither with genomic DNA nor with up to 10^6 copies of the plasmids.

Sequence analysis, ORF designation, and phylogenetic tree construction

PCR product purification and direct sequencing with dye terminator chemistry as well as nucleotide and amino acid sequence analysis were performed as described (Goltz et al., 2002). The following criteria were chosen for the ORF analysis: (i) presence of a typical translational start and stop signal, (ii) a minimal length of more than 60 amino acids, (iii) the longer of two overlapping ORFs is significant, and (iv) ORFs are conserved in other herpesviruses or found in at least two different PCR isolates of a PLHV species. The orientation of the analyzed genome stretch and the nomenclature of ORFs were adapted to those of HVS and other rhadinoviruses (Albrecht et al., 1992). The ORFs unique to PLHV-3 (P3.1 to P3.4) or PLHV-2 (P2.1), which had no homologs in databases, were only preliminarily designated. Redesignation may be necessary after possible detection of additional unique ORFs between the ORFs 03 and the left ends of the genomes. Multiple sequence alignments and phylogenetic tree construction with neighbor-joining and parsimonial analysis were performed as reported by Zimmermann et al. (2001).

Abbreviations and nucleotide sequence accession numbers

The PLHV-3 and PLHV-2 sequences determined in this study were deposited in the GenBank nucleotide sequence database (PLHV-3 from sample no. 1412, 25457 bp, Accession No. AY170315; PLHV-3 from sample no. 489, 60326 bp, Accession No.AY170316; PLHV-2 from sample no. 546, 32480 bp, Accession No. AY170314; PLHV-3 from sample no. 568, 59673 bp, Accession No. AY170317).

Abbreviations and accession numbers for the sequences used in Table 1 and Fig. 2 are AIHV-1 (alcelaphine herpesvirus 1) (complete genome [cg], Accession No. AF005370); BoHV-4 (bovine herpesvirus 4) (cg, Accession No. AF318573); CalHV-3 (callithrichine herpesvirus 3) (cg, Accession No. AF319782); CeHV-15 (cercopithecine herpesvirus 15 = rhesus monkey lymphocryptovirus) (cg, Accession No. X00784); EBV (Epstein-Barr virus) (cg, Accession No. AY037858); EHV-2 (equine herpesvirus 2) (cg, Accession No. U20824); HHV-8 (human herpesvirus 8) (cg, Accession No. U75698); HVS (herpesvirus saimiri or saimiriine herpesvirus 2 [SaHV-2]) (cg, Accession No. X64346); HVA (herpesvirus ateles or ateline herpesvirus 3 [AtHV-3]) (cg, Accession No. AF083424); MHV-68 (murine gammaherpesvirus 68) (cg, Accession No. U97553); PLHV-1 (porcine lymphotropic herpesvirus 1) (partial genome, Accession No. AF478169); RRV (rhesus monkey rhadinovirus) (cg, Accession No. AF029302). DPOL and gB sequences: BLHV (bovine lymphotropic herpesvirus) (DPOL, Accession No. AF327830; gB, Accession No. AF327832); CprHV-2 (caprine herpesvirus 2) (gB and DPOL, Accession No. AF283477); OvHV-2 (ovine herpesvirus 2) (DPOL, Accession No. AF327831; gB, Accession No. AF327833).

Purification of B-, T-, and myeloid cells from porcine blood

Blood samples were collected in EDTA-coated tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation on Ficoll-Paque (Pharmacia). Cells were washed three times in PBS containing 1% fetal calf serum. Remaining red blood cells were lysed with 155 mM ammonium chloride, 10 mM potassium hydrogencarbonate and 1 mM EDTA, diluted 1:1 with PBS, for 20 min at 4°C after the first washing step. B-cells, T-cells, and myeloid cells were purified by magnetic separation (MACS, Miltenyi BiotecCells). Cells (5×10^7 to 5×10^8) were incubated with 300 ng goat gammaglobulin on a rotating wheel at 4°C for 20 min to block unspecific binding of the antibodies used for the purification of the subpopulations. Mouse-anti-immunoglobulin (diluted 1:20; Biermann, Germany), mouse-anti-CD3 (0.1 μg/10^6 cells; Southern Biotechnologies, USA), or mouse-anti-SWC3 (diluted 1:50; kindly provided by A. Saalmüller, BFAV Tübingen) were added at 4°C for 30 min for selection of B-cells, T-cells, or myeloid cells, respectively. Unbound antibody was removed by washing twice in ice-cold PBS/1% fetal calf serum. Goat anti-mouse IgG Microbeads (Miltenyi Biotec) were then added at 4°C for 30 min. The cells were washed twice and applied to the MACS columns according to the manufacturer's instructions.

To determine the purity of the PBMC subpopulations, they were incubated with a FITC-labeled goat anti-mouse IgG antibody (diluted 1:250; Jackson ImmunoResearch), washed, and examined by flow cytometry (FACSCalibur, Becton Dickinson) after counterstaining with propidium iodide. For further analyses, only cell preparations of pigs were used in which all three subpopulations had a purity of at least 95%.

Real-time PCR analysis of purified PBMC subpopulations

For every pig four samples were examined (unsorted PBMC, Ig^+-cells, $CD3^+$-cells, $SWC3^+$-cells). For subsequent standardization of the four samples, these were tested in the β-actin assay first. The sample with the lowest C_t value (i.e., containing the highest amount of DNA and the lowest amount of PCR inhibitors) was chosen to calculate the difference to the other three C_t values (ΔC_t). These differences were then used for correcting the results of the PLHV assays. Based on the theory that the copy number of an amplimer doubles with every cycle during the run, the multiplication of the copy number indicated by the thermal cycler with $2^{\Delta C_t}$ gives the copy number which is leveled to the sample with the lowest C_t value.

Cell lines

The porcine B-cell line L23 was obtained from the European Collection of Cell Cultures (ECACC), Salisbury, UK, and maintained according to the supplied instructions. The EBV-positive cell line B95-8 and the human T-cell line Molt 4 were kindly provided by K. Borchers and K. Rokos, respectively, and both maintained in RPMI 1640/10% FCS.

Gardella gel electrophoresis, Southern blotting, and hybridization

Gardella gel electrophoresis was performed as described (Gardella et al., 1984). After the gel run, the DNA was depurinated with 0.25 N HCl, denatured with 0.5 N NaOH/1.5 M NaCl, transferred by vacuum blotting to a positively charged nylon membrane in the presence of 1 M ammonium acetate, and crosslinked. Hybridization was performed using digoxigenin-labeled probes. Unbound probe was removed with the Roche DIG Wash and Block Buffer Set according to the manufacturer's instructions. CDP-Star was used as a substrate and chemiluminescence was visualized by exposing the membrane to Lumifilm (Roche).

Generation of hybridization probes

For EBV detection, two probes were generated (PCR DIG Probe Synthesis Kit, Roche) from the gB and the DPOL gene, using purified viral DNA. For PLHV-3 detection, two cloned PCR products of the gB gene served as templates for DIG probe synthesis. Furthermore, two (semi-) nested PCR products were generated, spanning the ORFs 03–06 (3.7 kbp) and ORFs 21–23 (3.2 kbp). They were purified after agarose gel electrophoresis (Eppendorf

PerfectPrep Gel Cleanup) and labeled by random priming (DIG-High Prime, Roche). The PCR-generated probes were purified using Microspin S-400 HR columns (Pharmacia, Sweden). For annealing all probes were used in a total concentration of about 10 ng/ml hybridization solution.

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