Characterization of the DNA polymerase loci of the novel porcine lymphotropic herpesviruses 1 and 2 in domestic and feral pigs

Sven Ulrich, Michael Goltz and Bernhard Ehlers

Robert Koch-Institut, Nordufer 20, 13353 Berlin, Germany

Two novel porcine gammaherpesviruses, porcine lymphotropic herpesviruses 1 and 2 (PLHV-1 and -2), have been detected by amplification of short DNA polymerase (DPOL) sequences from blood and spleen of domestic pigs while searching for unknown herpesviruses in pigs as possible risk factors in xenotransplantation. In the present study, the DPOL genes of the two viruses and the open reading frames (ORFs) that follow in the downstream direction were amplified by PCR-based genome walking from adaptor-ligated restriction fragment libraries of porcine spleen samples. The sequences determined for the two PLHVs exhibited a very low G+C content (37 mol%) and a marked suppression of the CpG dinucleotide frequency. The DPOL proteins encoded were 95% identical and showed a close relationship (60% identity) to the DPOL protein of a ruminant gammaherpesvirus, alcelaphine herpesvirus 1 (AlHV-1). This was confirmed by phylogenetic analyses of the conserved regions of the two PLHV DPOL proteins. The PLHV ORFs downstream of DPOL exhibited 83% identity to each other and >50% similarity to ORF A5, the position equivalent of AlHV-1. From these data, the PLHVs can be firmly classified to the subfamily Gammaherpesvirinae. To find a natural reservoir for the PLHVs, organs of feral pigs were screened with five different PCR assays, targeting either the DPOL gene or 3′-flanking sequences. In all samples, PLHV sequences were detected that originated predominantly from PLHV-2, suggesting the possibility of virus transfer between feral and domestic pig populations.

Introduction

Xenotransplantation, the transplantation of animal organs into humans, is proposed as a potential solution for the constantly increasing organ shortage in allotransplantation, and domestic pigs are currently favoured as the donor animals (Ye et al., 1994; Fishman, 1994). However, xenotransplantation involves the risk of inadvertently transferring animal pathogens to the immunocompromised organ recipient (Michaels, 1997; Fishman, 1997). This risk is not merely hypothetical, since several viruses have been reported to cross barriers between animals and humans (Breman et al., 1980; Holmes et al., 1995; Gao et al., 1992, 1999). Most recently, a previously unknown porcine paramyxovirus caused the deaths of more than 100 people in Malaysia (Enserink, 1999). Furthermore, porcine endogenous retroviruses were reported to grow on cells of human origin (Patience et al., 1997; Martin et al., 1998) and simian retroviral sequences were detected in human recipients of baboon liver transplants (Allan et al., 1998). Therefore, recipients of xenotransplants have to be carefully monitored for transmission of animal viruses. However, viruses so far unknown would be excluded from this monitoring process for the lack of detection methods and would therefore be even more difficult to control. For this reason, we have previously begun a search for unknown herpesviruses in domestic pigs, because herpesviruses are frequently transmitted or reactivated in allotransplantation with resulting clinical complications (Britt & Alford, 1996; Feranchak et al., 1998; Hudnall et al., 1998; Kadakia, 1998). We have analysed porcine blood and tissues samples with a PCR assay that detects herpesvirus DNA polymerase (DPOL) sequences universally (Ehlers et al., 1999a). With this technique, short
sequences of two novel porcine herpesviruses with high similarity to gammaherpesviruses have been identified. They have been named porcine lymphotropic herpesvirus 1 (PLHV-1) and 2 (PLHV-2) (Ehlers et al., 1999b).

The goal of the present study was to characterize the genomes of the PLHVs further and to base their phylogenetic relationship and classification on analyses of complete genes. Furthermore, feral pigs (Sus scrofa) were analysed for the presence of PLHVs to find a natural reservoir.

Methods

**Collection of porcine blood and organ samples and preparation of DNA.** Blood and spleen samples were collected from commercial pig herds in Brandenburg, Germany. Bone marrow samples of feral pigs were collected from hunted animals in Germany. Total DNA was prepared by the QiAmp Blood and Tissue kits (Qiagen).

**Genome walking and cloning procedures.** PLHV sequences extending from the published PLHV sequences (Ehlers et al., 1999b) into unknown flanking regions were amplified by Genexpress GmbH (Berlin) on the basis of the Universal GenomeWalker kit from Clontech (Siebert et al., 1995). Briefly, separate aliquots of PLHV-1- and PLHV-2-infected pig samples as well as two samples of non-infected pigs were digested with six restriction enzymes. Each batch of digested genomic DNA was then ligated separately to an adaptor. With these adaptor-ligated libraries, nested PCR was performed by using an outer adaptor primer (AP1) and an outer gene-specific primer (GSP1) in first-round PCR and the inner primers AP2 and GSP2 in second-round PCR. The amplicons obtained were ligated into the plasmid pCR-Script Amp SK+ (Stratagene) and transformed into Epicurian Coli XL10-Gold Kan ultracompotent cells (Stratagene). Specific clones were identified by PCR with the primers AP2 and GSP2.

**PCR applications and nucleotide sequence determination.** In PCR analyses of PLHV-containing feral pig samples, both PLHVs were amplified with the primer pair 170-S/170-AS. PLHV-1 was amplified selectively with the primer pairs 170-S/160-AS or 213-S/215-AS and PLHV-2 with the primer pair 208-S/212-AS. All amplified regions are located inside the DPOL gene (Ehlers et al., 1999b). Primer pair 278-S/276-AS amplified a region of both PLHVs outside the DPOL gene (483 bp), with primer 278-S (′5′GGAATATGATGCCCTTATTAGGTTTTGTT-3′) binding to the intergenic region between DPOL and ORF A5 and primer 276-AS (′5′ATGGGGCCATTCCACCTACTTT3′) binding to the 5′ part of ORF A5. The locations of all amplified regions are depicted in Fig. 2(a).

For the PCR analyses of PLHV-containing domestic pig samples shown in Fig. 1, the PLHV-1-specific primer pair 170-S/170-AS and the PLHV-2-specific primer pair 280-S/280-AS (′5′CTCTATCATCCTACCATATGATTGC3′) and 280-AS (′5′GGCTATGTTGTATCCCGTAATAATC3′) were used.

Amplification of porcine cytomegalovirus (PCMV) sequences, the primers 199-S (′5′ACGGAGAGATATTTGGCGGTA3′) and 199-AS (′5′CTCTAGACGAAAGGACATTGTTGATA3′) were selected on the basis of the unpublished partial DPOL sequence of PCMV, strain B6, deposited in GenBank by F. B. Widen, M. Banks & P. J. Lowings (accession number AY122640). With these primers, an amplicon of 340 bp was obtained from PCMV strain B6 (kindly provided by M. Banks, Addelstone, UK). Amplicons of identical size were obtained from German spleen samples. Their sequences were 99% identical to the B6 sequence and the data were deposited in GenBank (accession no. AF191044).

The absence of PCR inhibitors in samples was tested by PCR with specificity for a conserved region of the vertebrate cytochrome B gene (Kocher et al., 1989).

The reactions were set up as described previously (Ehlers et al., 1999b). Amplifications were performed after complete activation of the DNA polymerase AmpliTaq Gold (Moretti et al., 1998) for 12 min at 95 °C and then cycled 40 times through 30 s denaturation at 95 °C, 30 s annealing at 55 °C (primers 170, 170-AS or 213-S/215, 278/276 and 280) or 58 °C (primers 208/212) and 30 s extension at 72 °C, followed by a final extension step at 72 °C for 10 min. PCR products were sequenced directly as described previously (Ehlers et al., 1999b).

**Nucleotide and protein sequence analysis.** Multiple sequence alignments were performed with the clustalW module of MacVector (version 6.01, Oxford Molecular Group). Protein pair distances were calculated with the MegAlign module of DNAStar (version 3.17). Phylogenetic analysis was based on a multiple amino acid alignment from which gaps or insertions unique to a particular species had been removed. The remaining conserved regions were concatenated (McGeoch et al., 1995) and subjected to phylogenetic tree construction by using the programs Protpars or Protdist and Neighbor from the PHYLIP package (Felsenstein, 1985, 1993). The trees were evaluated statistically by using 1000 bootstrap samples.

**Nucleotide sequence accession numbers.** The sequences from PLHV-1 (4643 bp), PLHV-2 (4642 bp) and PCMV (290 bp) determined in this study were deposited in the GenBank nucleotide sequence database under accession numbers AF191042 (PLHV-1), AF191043 (PLHV-2) and AF191044 (PCMV).

**Results**

The porcine gammaherpesviruses PLHV-1 and PLHV-2 have been detected previously by amplification of short viral DPOL sequences (464 and 423 bp, respectively) from blood and spleen samples of commercial domestic pigs (Ehlers et al., 1999b).
We now characterized the PLHV genomes to a larger extent by using a PCR-based genome walking technique on PLHV-containing porcine DNA.

Selection of PLHV-infected pig organs for PCR-based genome walking

To select the template material for the walking approach, we tested 27 spleen and 95 blood samples for the presence of PLHV-1 and PLHV-2 with the primer pairs 213-S/215-AS and 208-S/212-AS, respectively. Samples with strong PCR reactions for one of the PLHV species were re-examined by template dilution and subsequent PCR to estimate the PLHV copy number per ng total DNA. Additionally, we attempted to exclude samples containing PCMV by the use of the PCMV-specific primer pair 199-S/199-AS. Since we had previously found PCMV [but not pseudorabies virus (PRV)] in porcine spleen samples with degenerate primers (Ehlers et al., 1999b), we wanted to avoid a possible mixing of PLHV sequences with PCMV sequences generated in the genome walking process.

In this screening, no samples with a sufficient copy number of either PLHV-1 or PLHV-2 but negative for PCMV could be found. The reason for this was primarily the high prevalence of PCMV in the spleen samples (~90%). Blood samples had a low prevalence of PCMV (~15%) but also mostly had insufficient copy numbers of the two PLHVs. Therefore, a PLHV-1-positive spleen sample (#56) was selected that contained <100 PLHV-1 genomes per ng total DNA (<1 genome per cell) and was negative for PLHV-2. Likewise, a PLHV-2-positive spleen sample (#489) was selected that contained <10 PLHV-2 genomes per ng total DNA (<0.1 genomes per cell) and was negative for PLHV-1.

PLHV genome walking with adaptor-ligated restriction fragment libraries

The genome walking was based on a nested PCR approach, with adaptor-ligated genomic DNA fragments from the selected pig spleens #56 and #489, as described in Methods. Overlapping amplicons of 0.4–2 kbp were obtained, spanning a total of 5.5 kbp of PLHV-1 and 2.8 kbp of PLHV-2. The amplicons were cloned in E. coli by using the vector pCR-Script and then sequenced. Cloning and sequencing were repeated once or twice for identification of polymerase errors in individual clones. PCR was performed on the spleen samples #56 and #489 with specific primers derived from these sequences. The resulting amplicons were sequenced and compared with the walking-derived sequences. Upstream of the walking-derived region of PLHV-2, the walking process failed. Therefore, additional sequences of PLHV-2 were amplified by PCR only, by using sense primers derived from the PLHV-1 sequence and antisense primers from the PLHV-2 sequence. This approach was successful with some of the PLHV-1 primers because of the similarity of PLHV-1 and -2, shown in detail below. Consensus sequences of 4643 and 4642 bp, with 4- to 10-fold redundancy, were determined for PLHV-1 and PLHV-2, respectively, and used for further analyses. In a first step, the sequences were compared with the complete DPOL gene and adjacent downstream sequences of PCMV, since samples #56 and #489 contained not only PLHV but also PCMV. No significant stretches of identity were found in comparison with the PCMV sequences obtained from sample #489 and from the British PCMV strain B6 (M. Goltz & B. Ehlers, unpublished data) (not shown). Secondly, we analysed several regions of the DPOL genes of PLHV-1 and
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Fig. 3. Multiple amino acid alignment of the DPOLs of PLHV-1, PLHV-2 and AlHV-1. The two PLHV DPOLs are compared with their closest relative, the DPOL of AlHV-1. Dots indicate identical amino acids and dashes indicate gaps. The exonuclease domains (EXO I–III) and polymerization domains (Motif A–C) of the DPOL proteins are indicated by shading. The AlHV-1 DPOL was derived from the complete AlHV-1 genome sequence (accession no. AF005370; start of the DPOL ORF at nucleotide 19428).

-2 in additional blood and spleen samples. Identical amplicons and sequences were obtained for each PLHV species, which revealed the absence of PLHV sequence variation in samples of different origin. This is demonstrated by PCR results obtained with primer pairs that detect PLHV-1 or PLHV-2 differentially (Fig. 1).

PLHV open reading frame (ORF) and nucleotide composition analyses

In an ORF analysis, the 4643 bp PLHV-1 sequence was found to span the 3′ end of the glycoprotein B gene (184 bp), the complete ORF of DPOL (3012 bp) and an additional ORF (975 bp). The 4642 bp PLHV-2 sequence contained 184 bp of the 3′ end of the glycoprotein B gene, the complete DPOL ORF (3003 bp) and an additional ORF of 912 bp (Fig. 2a). Both sequences exhibited very low G+C contents of 37 mol% and a marked suppression of the CpG dinucleotide frequency with a concomitant increase of TpG and CpA (Fig. 2b). These characteristic dinucleotide frequencies have been found in several A+T-rich lymphotropic herpesviruses (Albrecht et al., 1992; Ensser et al., 1997; Virgin et al., 1997).

Analysis of the DPOL genes

The DPOL ORFs of PLHV-1 and PLHV-2 (3012 and 3003 bp, respectively) differed at 194 nucleotide positions, corresponding to an identity of 93%. The deduced amino acid sequences showed 50 amino acid differences, corresponding to an identity of 95% (Fig. 3). These differences confirmed our previous suggestion (Ehlers et al., 1999b) that the PLHVs are distinct, closely related species rather than strains of the same species. The PLHV-1 and PLHV-2 DPOLs (1004 and 1001 amino acids, respectively) were found to be the shortest herpesvirus DPOLs described so far, compared with those of other herpesviruses (1009–1246 amino acids). Both polymerases contained the conserved exonuclease (EXO I–III) and polymerization (motifs A–C) domains of DNA-dependent DNA polymerases of eukaryotic viruses (Knopf, 1998; Blanco et al., 1991) (Fig. 3).

Both PLHV DPOLs were closely related to the DNA polymerases of other gammaherpesviruses, in particular that of alcelaphine herpesvirus 1 (AlHV-1; Ensser et al., 1997). The PLHV DPOL genes were 60% identical to AlHV-1 DPOL. This identity is 8% lower than that reported previously (Ehlers et al., 1999b), since we analysed only a short, highly conserved region of DPOL in that study. Phylogenetic analysis was used to confirm the relationships observed. In the resulting phylogenetic tree, the herpesvirus subfamilies were separated with high bootstrap scores. In the cluster of the Gammaherpesvirinae, the PLHVs showed the shortest genetic distance to AlHV-1 (Fig. 4). The use of parsimony analysis gave a similar result (not shown).

Analysis of the ORFs downstream of the DPOL genes

The PLHV-1 and -2 proteins encoded by the ORFs downstream of the DPOL gene spanned 325 and 304 amino acids, respectively, and exhibited 87% similarity (83% identity). Both ORFs appeared to be homologues of the positional equivalent in the AlHV-1 genome (ORF A5, 302 amino acids), since the deduced proteins exhibited similarities of 50% (PLHV-1) and 53% (PLHV-2) to A5. Less similarity was found to the E6 protein of equine herpesvirus 2 (EHV-2) and the BILF1 protein of Epstein–Barr virus (EBV). Like A5, E6 and BILF1, the two PLHV proteins contained seven distinct hydrophobic regions representing putative transmembrane domains (not shown). They may function as G protein-coupled receptors, as discussed previously for A5 of AlHV-1 (Ensser et al., 1997).

Analysis of feral pigs for the presence of PLHV sequences

We next investigated whether a natural reservoir for the PLHVs exists in feral pigs. For this purpose, bone marrow samples from 19 feral pigs were analysed with different PCR
Contiguous sequences spanning approximately 4–6 kbp of each genome could be successfully amplified by genome walking from spleen samples that contained less than one viral genome per cell. The sequences were shown to contain the 3′ end of the glycoprotein B gene, the complete DPOL gene and an ORF with homology to the A5 gene of AlHV-1. This ORF exhibited low G+C content and the markedly reduced CpG frequency of the PLHV genomes group the PLHVs with several members of the genus Rhadinovirus such as herpesvirus saimiri (HVS or SaHV-2), murine gammaherpesvirus-68 (MHV-68) and AlHV-1, which exhibit low G+C contents of 35 (HVS) and 46 mol% (AlHV-1, MHV-68) as well as global CpG suppression (Albrecht et al., 1992; Ensser et al., 1997; Virgin et al., 1997). This characteristic dinucleotide frequency was suggested to be an indicator of the state of latency of A+T-rich lymphotropic herpesviruses (Hones et al., 1989).

As proposed previously (Ehlers et al., 1999b), PLHV-1 and PLHV-2 are different species rather than merely strains of the 170/160 (but not with the probably less-sensitive pair 213/215). Therefore, sample #562 appeared to be doubly infected (Table 1). These results were confirmed by sequence analysis. The sequences obtained from the 18 PLHV-2- and the two PLHV-1-containing samples showed 100% identity to the DPOL genes of PLHV-2 and PLHV-1, respectively, from domestic pigs. These data suggest that the same virus species infect domestic as well as feral pigs.

To provide more evidence for this hypothesis, we also compared the feral and the domestic PLHVs in a region of lower conservation outside the DPOL gene, by using the primer pair 278-S/276-AS (Fig. 2a). All bone marrow samples gave positive reactions (Table 1). Sequence analysis confirmed the presence of PLHV-2 in 18/19 samples and of PLHV-1 in sample #555, without any differences from the PLHV sequences found in domestic pigs. Only the PLHV-2 sequence was obtained from the doubly infected sample #562, indicating the particular presence of PLHV-2.

From the sequence analysis of > 700 bp of all feral PLHV isolates, we concluded that the PLHVs are infectious for domestic as well as feral pigs, with a particularly high prevalence of PLHV-2 in feral pigs.

### Discussion

The genetic data reported in this study provided compelling evidence for the existence of the novel porcine lymphotropic gammaherpesviruses PLHV-1 and PLHV-2. Contiguous sequences spanning approximately 4–6 kbp of each genome could be successfully amplified by genome walking from spleen samples that contained less than one viral genome per cell. The sequences were shown to contain the 3′ end of the glycoprotein B gene, the complete DPOL gene and an ORF with homology to the A5 gene of AlHV-1. Comparison of the PLHV DPOLs with those of other herpesviruses confirmed our previous observation (Ehlers et al., 1999b) that the PLHVs are closely related to gammaherpesviruses, in particular to AlHV-1. Furthermore, counterparts of the PLHV homologue of AlHV-1 A5 could be found in only two other gammaherpesviruses, EHV-2 and EBV. Therefore, PLHV-1 and PLHV-2 can be classified as members of the subfamily Gammaherpesvirinae. In addition, the low G+C content and the markedly reduced CpG frequency of the PLHV genomes group the PLHVs with several members of the genus Rhadinovirus such as herpesvirus saimiri (HVS or SaHV-2), murine gammaherpesvirus-68 (MHV-68) and AlHV-1, which exhibit low G+C contents of 35 (HVS) and 46 mol% (AlHV-1, MHV-68) as well as global CpG suppression (Albrecht et al., 1992; Ensser et al., 1997; Virgin et al., 1997). This characteristic dinucleotide frequency was suggested to be an indicator of the state of latency of A+T-rich lymphotropic herpesviruses (Hones et al., 1989).

As proposed previously (Ehlers et al., 1999b), PLHV-1 and PLHV-2 are different species rather than merely strains of the
same species, since the two viruses exhibited 50 amino acid differences in the DPOL genes. In contrast, strains of the same gammaherpesvirus species appear to contain no mutations or only a few, mostly silent, mutations in their DPOL genes, as shown for the complete DPOL genes of different strains of human herpesvirus-8 (HHV-8) (Neipel et al., 1998), Tupaia herpesvirus (Springfield et al., 1998) and the recently discovered rhesus monkey rhadinovirus (RRV) (Searles et al., 1999). The same observation was made with partial DPOL sequences of strains of several other herpesvirus species from different subfamilies (VanDevanter et al., 1996; Ehlers et al., 1999a). We therefore propose the taxonomic names suid herpesvirus-3 (SuHV-3) and suid herpesvirus-4 (SuHV-4) for PLHV-1 and PLHV-2.

Both PLHVs were found in organs of 19 feral pigs, with an exceptionally high prevalence of PLHV-2. No differences were found between the domestic and feral pig sequences, suggesting the presence of the same virus species in domestic and feral pigs. Therefore, transfer of these viruses between feral and domestic pig populations might be possible, as has been reported for PRV (Capua et al., 1997) and classical swine fever virus (Stadejek et al., 1997). This underlines the need for the isolated breeding of pigs intended for use as organ donors in xenotransplantation.

The discovery of the porcine lymphotropic gammaherpesviruses PLHV-1 and PLHV-2 was the result of a search for unknown herpesviruses in order to unravel risk factors in xenotransplantation. Therefore, the possible pathogenic potential of the PLHVs, not only for pigs but especially for immunocompromised humans, is of concern and should be analysed further. An initial step in this direction was the comparative analysis of the genetic data acquired so far. It revealed a close relationship of the PLHVs to the ruminant gammaherpesviruses AlHV-1, on the basis of analysis of the complete DPOL gene and the A5 ORF (this study), and ovine herpesvirus-2 (OvHV-2) and bovine lymphotropic herpesvirus (BLHV), on the basis of analysis of partial DPOL sequences (Ehlers et al., 1999b). AlHV-1 and OvHV-2 cause malignant catarrhal fever, a lymphoproliferative disease of cattle with high mortality (Reid & Buxton, 1989), and BLHV has been discussed as a cofactor in bovine leukaemia (Rovnak et al., 1998). This suggests possible pathogenicity of the PLHVs. However, no link has yet been discovered between the PLHVs and lymphoproliferative or neoplastic diseases. Growth of the PLHVs in cell culture is a prerequisite for such studies, and such culture experiments are currently in progress.

A common feature of gammaherpesviruses is the presence of homologues of cellular genes. They have been found or predicted to modulate the cell cycle, to regulate apoptosis, to interfere with immune functions or to function in cytokine signal transduction. These genes are probably part of the virus defence mechanisms against elimination by the host and therefore are likely to contribute to virus virulence (reviewed by Neipel et al., 1997; Simas & Efstratiou, 1998). For this reason, walking on the PLHV-1 genome is continuing to find such virus virulence genes that may contribute to the pathogenicity of the PLHVs for human organ recipients in xenotransplantation.

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