Detection of two novel porcine herpesviruses with high similarity to gammaherpesviruses

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Evidence for the existence of porcine gammaherpesviruses was obtained by PCR and sequence analysis. Initially, samples of peripheral blood mononuclear cells (PBMC), spleens, lungs, kidneys and livers of pigs from Germany and Spain were tested with a PCR assay which targets conserved regions of the herpesvirus DNA polymerase gene with degenerate and deoxyinosine-substituted primers. Amplicons of identical sequence were obtained from one spleen and two PBMC samples. This sequence showed a high percentage of identity with the DNA polymerase genes of herpesviruses of the oncogenic subfamily Gammaherpesvirinae. Alignment of amino acid sequences showed the highest identity values with bovine gammaherpesviruses, namely alcelaphine herpesvirus type 1 (68%), ovine herpesvirus type 2 (68%) and bovine lymphotropic herpesvirus (67%). Comparison with pseudorabies virus and porcine cytomegalovirus, which are the only porcine herpesvirus species presently known, showed values of only 41%. PCR analysis of PBMC (n = 39) and spleen (n = 19) samples from German pigs, using primers specific for the novel sequence, revealed a prevalence of 87 and 95%, respectively. In this analysis, three out of eight spleen samples from Spanish pigs were also positive. Subsequent sequencing of the amplicons revealed the presence of two closely related gammaherpesvirus sequences, differing from each other by 8% at the amino acid level. The putative novel porcine herpesviruses, from which these sequences originated, were tentatively designated porcine lymphotropic herpesvirus type 1 and type 2 (PLHV-1 and PLHV-2). When using pig organs for xenotransplantation, the presence of these viruses has to be considered.

Introduction

Transplantation of animal organs into humans (xenotransplantation) is considered to be a solution for the shortage of organs presently available for allotransplantation. However, the transfer of animal organs to humans has raised concerns about the possibility that animal donors might harbour microorganisms with pathogenic potential for the human recipient and the wider population. In the past, several pathogenic infectious agents have been transferred from non-human primates to men (Holmes et al., 1995; Gao et al., 1992). This is one of the reasons why non-human primates are considered to be unsuitable organ donors for xenotransplantation (Chapman et al., 1995). With pigs serving as donors, the risk of transferring diseases to humans is supposed to be markedly lower. A close association of men with pigs has existed over the centuries, and no serious infectious disease of pig origin has been observed in humans, with the exception of some influenza virus strains. Therefore, pig organs are currently favoured for transplants (Fishman, 1994). However, in xenotransplantation, recipients of organs would receive immunosuppressive treatment in order to prevent organ rejection (Brazelton, 1997). Under these conditions, swine pathogens and even non-pathogenic microorganisms present in pigs might adapt to the immunocompromised human recipient and cause disease or recombine with human viruses to create new pathogens. To avoid such epidemiological risks, pig breeds have to be as free as possible of potential pathogens in order to serve as source animals in xenotransplantation. It is therefore necessary to identify so far undescribed, potentially pathogenic viruses harboured by pigs in order to provide the basis for the development of surveillance methods.

We attempted to identify novel species of the virus family Herpesviridae because of their clinical importance during immunosuppressive treatment after allotransplantation.
(Fishman, 1997). Furthermore, although in humans and other mammals (monkeys, horses, cattle, rodents) several different herpesviruses had already been described (Roizman et al., 1995), only two herpesvirus species infecting pigs were known: pseudorabies virus (PRV; suid herpesvirus 1) and porcine cytomegalovirus (PCMV; suid herpesvirus 2). PRV is a member of the subfamily Alphaherpesvirinae. It causes encephalomyelitis and inflammation of the respiratory tract. Severe losses of young piglets occur in unvaccinated herds and many non-porcine mammals are also susceptible (Wittmann & Rziha, 1989; Mettenleiter, 1991). PCMV is a member of the subfamily Betaherpesvirinae. It is found in the respiratory tract of pigs and causes atrophic rhinitis (Ohlinger, 1989). A member of the herpesvirus subfamily Gammaherpesvirinae infecting pigs was not known. Therefore, we examined pigs for the presence of a gammaherpesvirus by a modified PCR assay which targets highly conserved amino acid motifs of the herpesvirus DNA polymerase with degenerate and deoxyinosine-substituted primers (VanDevanter et al., 1996).

Here, we present two new herpesvirus-related DNA polymerase sequences that indicate the existence of two novel members of the subfamily Gammaherpesvirinae in pigs. These putative viruses were tentatively designated porcine lymphotropic herpesvirus type 1 and type 2 (PLHV-1 and PLHV-2).

Methods

**Preparation of peripheral blood mononuclear cells and tissue DNA.** Blood samples were collected from commercial pig herds and spleens were from a slaughterhouse in Brandenburg, Germany, in 1998. Spleens, lymph nodes, kidneys, lungs and livers from pigs of commercial herds in Spain were kindly provided by M. Domingo (Universidad de Barcelona, Barcelona, Spain). Total DNA of pig peripheral blood mononuclear cells (PBMC) and of pig organs were prepared with the QiAamp Blood kit and QiAamp Tissue kit (Qiagen).

**Consensus PCR.** Consensus PCR was carried out as nested PCR with three degenerate primers (two sense primers and one antisense primer) in first-round PCR and two degenerate primers in second-round PCR as described by VanDevanter et al. (1996), with modifications as described below. The first-round primers DFA (5'-GAY TTY GCN AGY YTN TAY CC 3') (sense), ILK (5'-TCC TGG ACA AGC ARN YSG CNM TNA A 3') (sense) and KG1 (5'-GTC TTG CTC ACC AGN TCN ACN CCY TT 3') (antisense) were mixed with the deoxynosine-substituted primers I-DFA (5'-GAY TTY GCI AGY YTI TAY CC 3'), I-ILK (5'-TCC TGG ACA AGC AGC ARN YSG CIM TIA A 3') and I-KG1 (5'-GTC TTG CTC ACC AGI TCI CCY TT 3'). Likewise, the second-round primers TGV (5'-TGG TAC CGC TGG TAY GTG TGG GNJ TYN ACN GNJ GT 3') (sense) and IYG (5'-CAG AGA GTC GTG RTY NCC RTA DAT 3') (antisense) were mixed with the deoxynosine-substituted primers I-TGV (5'-TGG TAC CGC TGG TAY GGI TTY AGI GGI GT 3') and I-IYG (5'-CAG AGA GTC GTG RTY NCC RTA DAT 3').

PCR reactions were carried out with 50 ng PBMC or 100 ng tissue DNA as templates. In the second round of nested PCR, 1 µl first-round PCR reaction was used as template. Reaction mixtures (25 µl) contained 1 µM each PCR primer (Pharmacia Biotech), 200 µM each deoxy-nucleotide triphosphate, 0.7 U DNA polymerase AmpliTaq Gold, 2 mM MgCl₂, 2.5 µl 10 × GeneAmp PCR buffer II (PE Applied Biosystems) and 5% DMSO (Sigma–Aldrich). For thermal cycling, Perkin-Elmer 2400 thermocyclers and 0.2 ml thin wall tube strips were used. Cycling was performed with a time-release protocol which activates the inactive polymerase AmpliTaq Gold only partially before cycling. Full activation is then achieved during the first cycles (Kebelmann-Betzig et al., 1998). In first-round PCR, the reaction mixtures were kept at 95 °C for 3 min and then cycled 55 times with 20 s denaturation at 95 °C, 30 s annealing at 46 °C and 30 s strand extension at 72 °C, followed by a final extension step at 72 °C for 10 min. In second-round PCR, conditions were identical except that the annealing and extension times were reduced to 20 s.

For sequence analysis of the second-round PCR products, 1 µl PCR reaction was reamplified as described by VanDevanter et al. (1996).

**Specific PCR.** Specific primer combinations 160-S (5'-CAT CTG GTA TGC TGC CCT GTC T 3')/160-AS (5'-AAG GGT TTA TCA ATG CTG TTT GG 3'), 167-S (5'-CAG AAA GGA ATT AGC AGC ATG TGC T 3')/167-AS (5'-GAG GCA TAA AGC CAA CCT TAC AGA 3'), 170-S (5'-GCT GAC CCA AAG CTC AGG ACA ATT T 3')/170-AS (5'-TAT CGC CGT AGA TCA CCT TGA AGG G 3') and 175-S (5'-GTC ACT CTA CCC TAA TCC ATC AT 3')/175-AS (5'-GCA ACA CCA GTG AAC CCA TAC A 3') were used for amplification of 170, 252, 277 and 284 bp of the PLHV-1 DNA polymerase, respectively. Primer combinations 170-S/170-AS and 175-S/175-AS were also suitable for amplification of the PLHV-2 DNA polymerase.

Primer combinations 213-S (5'-TCC ATC ATG AAG ACC TGC ATA AA 3')/215-AS (5'-CCT TAC AGA TGG AAT GGA GAT CC 3') and 208-S (5'-CCT TCT TGT CGA AGT TGC TCA CA 3')/212-AS (5'-ACC TTG AAG GGT TTA TCA AAC AC 3') were designed on the basis of the sequence differences between PLHV-1 and PLHV-2 (Fig. 3a) for differential amplification of PLHV-1 (393 bp) and PLHV-2 (334 bp) fragments, respectively.

Amplification was performed after complete activation of the polymerase for 12 min at 95 °C and then cycled 42 times with 30 s denaturation at 95 °C, 30 s annealing at 51 °C (pair P-160), 55 °C (pair P-170, pair P-175, P-213/215), 58 °C (P-208/212) or 62 °C (pair P-167) and 30 s strand extension at 72 °C, followed by a final extension step at 72 °C for 10 min.

All DNA preparations from tissue and PBMC samples were tested for the absence of PCR inhibitors by PCR with primers 145-S (5'-TCT GCC CTA TCA ACT TCT GAT GGT A 3') and 145-AS (5'-AAT TTG CGC GCC TGC TGC CTT CCT T 3'), using the above PCR protocol at an annealing temperature of 64 °C (amplicon, 137 bp). These primers universally detect eukaryotic DNA (Gonzalez & Schmickel, 1986; Barker et al., 1988).

**Sequence analysis.** PCR products were sequenced directly. PCR primers remaining in the PCR reaction mixture were removed by MicroSpin S-300 or S-400 HR spin columns according to the manufacturer's instructions (Pharmacia Biotech). Sequencing of PCR products was performed by the deoxynucleotide termination cycle sequencing method, using the Prism BigDye Ready Reaction Terminator Cycle Sequencing kit (PE Applied Biosystems). Reaction and cycling conditions were chosen according to the manufacturer's protocol. Sequencing reactions were run on ABI 377 or ABI 310 automated sequencers (PE Applied Biosystems) and subsequently analysed by the Sequencing Analysis, Sequence Navigator and AutoAssembler software packages (PE Applied Biosystems), MacVector (Oxford Molecular Group) and the Heidelberg Unix Sequence Analysis Resources (HUSAR) software package (Genetics Computer Group, Wisconsin and German Cancer Research Centre, Heidelberg).

**Sequence alignments and phylogenetic analysis.** Sequences were aligned with the CLUSTAL W module of MacVector. Phylogenetic
trees were derived from amino acid sequence alignments using the programs Protpars or Protdist and Neighbour from the PHYLIP program package (Felsenstein, 1985). The trees were statistically evaluated using 1000 bootstrap samples.

Nucleotide sequence accession numbers. The PLHV-1 and PLHV-2 DNA polymerase sequences were deposited in the GenBank nucleotide sequence database (accession nos AF118399 and AF118401).

Results

Amplification of gammaherpesvirus-like DNA polymerase sequences with degenerate and deoxyinosine-substituted primers

A consensus PCR assay targeting regions of conserved amino acid motifs in herpesvirus DNA polymerase genes was used to search for novel herpesvirus species in pigs. Consensus PCR was carried out in a nested format as described by VanDevanter et al. (1996) who amplified the polymerase genes of 22 known herpesviruses. Because several reports had described the good performance of deoxyinosine-substituted primers in targeting related (non-herpesvirus) sequences (Rossolini et al., 1994; Cassol et al., 1991; Knoth et al., 1988), we mixed every primer with a second deoxyinosine-substituted primer, as described in Methods, to increase the potential of this PCR system for the detection of novel herpesviruses. By using these primer mixtures, we had previously amplified the DNA polymerase genes of six human, four bovine, five equine, two porcine and two avian herpesviruses from purified viral DNA and biopsy specimens. In addition, we had found sequence evidence for new gammaherpesviruses in PBMC of wild and zoo equids (Ehlers et al., 1999).

In order to assay for a gammaherpesvirus in pigs, we initially analysed samples from two distant geographic origins with consensus PCR (primers DFA/1-DFA, ILK/1-ILK and KG1/I-KG1 in first-round PCR; and primers TGV/1-TGV and IYG/I-IYG in second-round PCR). PBMC collected from eight pigs of a herd in Brandenburg (Germany) and samples of spleen, lymph nodes, kidneys, livers and lungs of ten pigs originating from Spain were tested. From two German blood samples and a spleen sample from one Spanish animal, PCR bands of 231 bp were generated. Furthermore, amplicons of 228 bp were obtained from one spleen and three lung samples. Sequence analysis of the 228 bp amplicons (175 bp excluding primed regions) and subsequent FASTA analysis in the GenBank database showed 98% identity with the unpublished partial DNA polymerase sequence of PCMV deposited in GenBank by F. B. Widen, M. Banks & P. J. Lowings (accession no. A]222640). The PCMV origin of the sequence was confirmed by personal communication (M. Banks, Central Veterinary Laboratory, Addlestone, UK). The amino acid sequences predicted from the PCMV sequence and the nearly identical 175 bp sequence detected in this study revealed in pairwise alignments with herpesviral DNA polymerases the highest similarity to members of the Betaherpesvirinae (data not shown).

Analysis of the 231 bp sequence from spleen and PBMC samples showed strong similarity to several members of the subfamily Gammaherpesvirinae, with identity values of up to 64%. Also, the amino acid sequence predicted from the 231 bp sequence revealed a close relationship to gammaherpesvirus DNA polymerases. Therefore, this sequence (178 bp excluding primed regions) was likely to originate from a novel herpesvirus which was tentatively named porcine lymphotropic herpesvirus type 1 (PLHV-1).

To extend the sequence information for PLHV-1, a semi-nested PCR was performed. The amplification product of PCR with the primer mixtures DFA/I-DFA and KG1/I-KG1 was used as a template for PCR with the sense-primer mixture DFA/I-DFA and a PLHV-specific antisense primer (170-AS) (Fig. 1). The sequence of the generated PCR amplicon (455 bp) was then combined with the 178 bp PLHV-1 sequence obtained initially. This resulted in a total sequence of 466 bp of the PLHV-1 DNA polymerase, excluding primed regions. To validate this sequence, PLHV-specific primer pairs (numbers 160, 167, 170 and 175) were used for generation of

Fig. 1. Comparison of the PLHV-1 DNA polymerase sequence with the corresponding sequence of AlHV-1. Amplification products of the PLHV-1 DNA polymerase gene were obtained from a porcine PBMC sample by consensus PCR and semi-nested PCR, as described in the text, and sequenced. The partial DNA polymerase sequence of AlHV-1 was taken from the AlHV-1 sequence deposited in GenBank (accession no. AF005370). Position 1 in this figure corresponds to position 21269 of the GenBank sequence. Identical nucleotides are marked with asterisks. Black triangles mark 3' ends of primers used for amplification of PLHV-1. The primer mixtures of first-round consensus PCR, DFA/I-DFA and KG1/I-KG1, bind 18 bp upstream and 211 bp downstream of the PLHV-1 sequence presented; this is indicated by the slashes.
Fig. 2. Multiple sequence alignment of predicted amino acids of the PLHV-1 and PLHV-2 DNA polymerases and those of other herpesviruses. The amino acid sequences of the PLHV-1 and PLHV-2 DNA polymerases were deduced from the sequences shown in Fig. 3(a) and aligned with the DNA polymerase sequences of known bovine (AlHV-1, BLHV, BHV-4), ovine (OvHV-2), human (EBV, HHV-8), monkey (HVS, RRV, RFHVMm, RFHVMn), murine (MGHV68) and equine (EHV-2) gammaherpesviruses, obtained from GenBank (see legend to Fig. 4 for definitions and accession nos). The sequences of the porcine alpha- and betaherpesviruses PRV (accession no. L24487) and PCMV (accession no. AJ222640) were included for comparison. Identical amino acids are shown in bold type on a dark grey background; similar amino acids are shown on a light grey background.

Pairwise alignments of the predicted amino acid sequence with herpesviral DNA polymerases revealed the highest similarity to bovine gammaherpesvirus polymerases. Identity...
Detection of novel porcine gammaherpesviruses

Values of 68% were found with the corresponding sequences of alcelaphine herpesvirus type 1 (AlHV-1) and ovine herpesvirus type 2 (OvHV-2). Comparison with the recently described bovine lymphotrophic herpesvirus (BLHV) (Rovnak et al., 1998) revealed 67% identity. In contrast, for the porcine alphaherpesvirus PRV only 41% identity was determined. Similar low percentage values were found for the sequences of porcine betaherpesvirus PCMV and other alpha- and beta-herpesviruses (<45%). A multiple amino acid sequence alignment of the PLHV-1 sequence with DNA polymerase sequences of 12 gammaherpesviruses as well as PCMV and PRV is presented in Fig. 2. It shows that the PLHV sequence is gammaherpesvirus-like.

**Survey of blood and spleen samples with specific PCR**

The PLHV-1-specific primers 170-S and 170-AS were used for analysis of additional porcine samples. PBMC from 42 pigs (sows, boars, piglets) obtained from seven different commercial herds and 19 spleen samples randomly collected from four different days from commercial slaughter in Brandenburg, Germany, were analysed. Amplicons were obtained from 88% (37 out of 42) of the PBMC samples and 95% (19 out of 20) of the spleen samples. In addition, three out of eight spleen samples of pigs originating from Spain were positive. Cell lines of suid origin (PS, PK-15) were negative (data not shown). Furthermore, template dilution experiments were performed with several blood and spleen samples. In two of these samples, at least 1000 copies of PLHV-1 DNA were found. PCR-negative samples remained negative after nested PCR with the primer combination 170-S/170-AS, which confirmed that the detected PLHV sequences were not part of the pig germ line.

**Detection and analysis of a second gammaherpesvirus-like sequence**

The amplicons from 17 PBMC and 14 spleen samples obtained with the primer pair 170-S/170-AS (227 bp without
The attempt to extend the sequence information for PLHV-2 with a semi-nested PCR approach similar to that used for amplification of PLHV-1 was not successful, probably because of poor binding of the DFA/I-DFA mixture to the PLHV-2 sequence. However, PLHV-2 could be amplified with the primer pair 175-S/175-AS (Figs 1 and 3a). We analysed two of the seven PLHV-2-positive samples and determined identical sequences, differing from the PLHV-1 sequence. In total, 423 bp of the PLHV-2 polymerase gene were obtained and compared with the PLHV-1 polymerase sequence. This revealed differences at 26 nucleotide positions (Fig. 3a).

**Specific, differential amplification of PLHV-1 and PLHV-2**

In order to detect each PLHV type differentially, we designed primer combinations targeting the sequence differences between PLHV-1 and PLHV-2. As shown in Fig. 3b, the primer combination 213-S/215-AS specifically amplified PLHV-1, whereas the primers 208-S and 212-AS were specific for PLHV-2. Again, the amplicons were verified by sequence analysis (data not shown). In summary, these results strongly indicated the existence of two different porcine gammaherpesviruses.

**Phylogenetic analysis of PLHV-1 and PLHV-2**

For phylogenetic analysis, a phylogenetic tree was constructed with the program Protpars of the PHYLIP package and statistically evaluated using 1000 bootstrap samples. In this analysis, the PLHV viruses clustered with the bovine gammaherpesviruses BLHV, AIHV-1 and OvHV-2 and showed a distance from each other that was similar to the distance between AIHV-1 and OvHV-2 (Fig. 4). This phylogenetic tree was also supported in analyses with the programs ProtDist and Neighbour (not shown).

**Discussion**

The search for herpesviruses in porcine blood and tissue samples from different geographic origins with a consensus PCR assay targeting the herpesviral DNA polymerase with degenerate and deoxyinosine-substituted primers indicated the existence of porcine gammaherpesviruses. Differential PCR and sequence analysis demonstrated the presence of two closely related, but different gammaherpesviruses (Fig. 3). The novel putative viruses from which these sequences originated were tentatively named PLHV-1 and PLHV-2.

The PLHV-1 and PLHV-2 sequences differed from each other by 26 out of 423 bases and had predicted proteins that differed by 8 out of 141 amino acids. Fourteen of these sequence differences were found in the region which is amplified by consensus PCR, between the binding regions of primers TGV and IYG (Figs 1 and 3a). In contrast, strains of a given herpesvirus species differ in this region by a maximum of two bases. This was reported for strains of HHV-1, HHV-6 and HHV-7 (VanDevanter et al., 1996) and was also found for strains of PRV, EHV-1 and BHV-1 (Ehlers et al., 1999). Therefore, it is likely that the PLHV-1 and PLHV-2 sequences...
originated from closely related, but different virus species rather than from two different strains of the same species.

Analysis of the partial DNA polymerase sequences of the PLHV by amino acid sequence alignments and phylogenetic analysis revealed a high similarity with the malignant catarrhal fever viruses of cattle, AlHV-1 and OvHV-2, and with the bovine lymphotropic gammaherpesvirus BLHV as shown in Figs 2 and 4, but a considerably lower relationship to the known porcine herpesviruses PRV and PCMV. These data were supported by preliminary sequence data obtained by genomic walking through the complete polymerase gene and the immediately adjacent glycoprotein B gene of PLHV-1 (data not shown).

The PLHV sequences were detected with the consensus PCR assay in blood and spleen samples but not in other organs. In additional analyses of approximately 60 porcine blood and spleen samples with non-degenerate primers, PLHV were detected in 80% of the samples. From these data, we assumed that these viruses are lymphotropic and ubiquitously present in commercial pig herds.

So far, nothing is known about the pathogenic potential of PLHV. The closely related bovine gammaherpesviruses AlHV-1 and OvHV-2, as well as a more distantly related primate gammaherpesvirus, herpesvirus saimiri, are classified as members of the genus Rhabdovirus. They are apathogenic in their natural hosts but cause serious lymphoproliferative diseases in other species (Reid & Buxton, 1989; Fleckenstein & Desrosiers, 1982). Accordingly, PLHV could be apathogenic in pigs but pathogenic in other species. Alternatively, it is possible that PLHV induces a disease in pigs that has remained unrecognized because of the usually short lifespan (6 months) of a commercial pig.

Even if PLHV are apathogenic in pigs, the transmission of PLHV after transplantation of pig organs to humans could have xenozoonotic potential, since recipients of xenografts will be heavily and continuously immunosuppressed (Brazelton, 1997). The viruses would be directly introduced into the body of the recipient via an infected organ, circumventing defence barriers like mucosal surfaces. This could favour the transmission between pigs and humans. In addition, PLHV viruses might recombine with the human herpesviruses HHV-6, HHV-7 and human cytomegalovirus, which are frequently reactivated in the post-transplantation stage (Fishman, 1997). Therefore, virus isolation and characterization are necessary to study the biology and the pathogenic potential of the PLHV viruses. Such data could be the basis for evaluation of the probability of PLHV transmission to humans and the possible consequences of such transmissions when using pig organs for xenotransplantation.

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