Polymerase chain reaction in detection of porcine endogenous retrovirus (PERV) from porcine tissues

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Abstract Pigs offer an unlimited source of xenografts for humans. The use of transplants from animal origin offers a potential solution to the limited supply of human organs and tissues. However, like many other mammalian species, pigs harbor porcine endogenous retrovirus (PERV), which are encoded in their genomic DNA and are assumed to have been integrated into the porcine germline. The ability of PERV to infect human cells *in vitro* has heightened safety concerns regarding the transmission of PERV to pig xenograft recipients. Porcine tissues were analyzed using validated assays specific for PERV: polymerase chain reaction (PCR) (for PERV DNA) and reverse transcriptase (RT)-PCR (for PERV RNA). PERV-specific gag sequences were found in the porcine heart tissue samples using DNA-PCR and RT-PCR. PCR is a rapid and specific test for the detection of PERV from xenografts. These findings have demonstrated that the presence of both DNA and RNA forms of PERV in porcine tissues needs to be carefully considered when the infectious disease potential of xenotransplantation is being assessed.

Keywords Polymerase chain reaction (PCR) · Porcine endogenous retrovirus (PERV) · Xenograft

Introduction

Xenotransplantation has been a dream of both science and practitioners to solve the problem of organ availability in order to save human lives. There are three classes of high-risk organisms that can potentially cause zoonoses: bacteria, fungi and parasites and viruses, especially retrovirus. Besides other bacteria, fungus and viruses, pigs harbor porcine endogenous retrovirus (PERV), which are encoded in their genomic DNA [1, 2] and assumed to be descendants of ancient viruses that became integrated into the host germline. PERV belongs to mammalian Type-C gamma retrovirus. Pig genomes have been shown to contain multiple copies of at least three distinct classes of endogenous type-C retroviruses, including full-length viral genomes, with distinct *env* genes, referred to as *PERV-A*, *PERV-B* and *PERV-C*. Two main types of pig retroviruses, PERV-A and PERV-B, which differ by 507 bases in their envelope (*env*) genes, are widely distributed in different pig breeds. PERV displays approximately 50 proviral integration sites in different breeds of pig genome. Host range analysis by the vector transduction assay has showed that PERV-A and PERV-B viruses have wider host ranges, including several human cell lines than PERV-C viruses, which infect only two pig cell lines and one human cell line. Two of the three identified receptor classes of PERV, distinguished by their envelope sequence and tropism, have been shown to be capable of replicating in human cells *in vitro* [3–6]. Clinical trials to date with live pig xenograft tissues include perfusion with pig livers or porcine hepatocytes as a bridging strategy for hepatic failure, use of pancreatic islet cells as a treatment for chronic diabetics and implantation of fetal neuronal tissue as a therapy for Parkinson's disease [7].

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PERV particles are spontaneously released in cultures of porcine peripheral blood lymphocytes (PBLs) and cell lines from a variety of porcine tissues including kidneys (PK15 MPK, PORC), lymph nodes (Shimozuma-1, 38A-1, Testes, ST-MO) and fallopian tubes (PFT). PERV from PK-15 cells can infect a variety of human cell lines *in vitro* such as kidney, lung, muscle and lymphoid cells, suggesting that these retroviruses may also be able to infect a spectrum of human tissues *in vivo* [8]. Therefore in this study porcine tissues to be used as xenografts were analyzed using PCR and RT-PCR for the molecular detection of PERV sequences.

Materials and methods

Cell lines

Cells and tissue culture supernatants used in the PCR and RT-PCR analyses were porcine kidney epithelial cell line; PK-15 obtained from National Centre for Cell Science Pune (NCCS) was used as a positive control for PCR. Human PBL samples were obtained by Ficoll–Hypaque (Sigma) centrifugation of ethylene diamine tetra acetic acid (EDTA)-preserved whole blood and was used as a negative control. All cell lines were maintained in minimal essential medium (MEM) (Gibco), supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1% nonessential amino acids, 100 U of penicillin per ml and 100 µg of streptomycin per ml.

Sample preparation for PERV proviral PCR assay

PCR assay targeting the gag gene region was developed to detect PERV sequence in aortic valve, pulmonary valve and heart muscle samples obtained from fresh porcine xenograft tissue. Porcine heart samples were collected from Veterinary College, Chennai and Meat Products of India, Kerala. The primers used were PRE TF 1, 5' CGG CAA GAG AAG AAT TTG ACT AAG ATC 3' and PRE TR 1, 5'CAG TTC CTT GCC CAG TGT CCT CTT 3', to amplify a 187 bp

gag sequence. Negative amplification controls for each assay included normal human PBL and a positive control included PK-15 cell lysates which were prepared and subjected to amplification. Preparation of genomic DNA from porcine tissues was done using a Qiagen Mini preparation kit and the PCR was performed with standard conditions of 1 min. at 94°C, 1 min. at 55°C and 1 min at 72°C for 35 cycles using 25 µl (5–10 µg) of DNA template in 100 µl reaction volumes containing 1X PCR reaction buffer (1.5 mM MgCl₂), 2.5 U Taq polymerase (Qiagen), 1.25 mM of each deoxynucleotide triphosphate (dNTP) and 100 ng of each oligomer (Qiagen). The PCR products (10 µl) were electrophoresed on 1.8% agarose gel. All PCR assays were performed following recommended precautions to prevent contamination.

RT-PCR analysis

We developed an RT-PCR assay to detect PERV RNA sequence directly in tissue samples. RNA was extracted from the samples using Ultra Clean Tissue RNA kit (Mobio Laboratories, CA). 25 µl of RNA extract was treated with 10 U of RNase-free DNase for 1 h at 37°C followed by inactivation of the DNase by boiling for 5 min. Reverse transcription was performed in 50 µl reaction volumes containing 100 ng of PRE TR1 primer, 10X RT buffer, 1.25 mM of each dNTP, 20 U of MMLV reverse transcriptase enzyme (Mobio Laboratories, CA). The reaction mixture was incubated at 37°C for 2 h. Control RT-PCR reactions that received no RT were included for each sample to confirm that a positive result was due to the presence of PERV RNA and not due to contamination with residual PERV genomic DNA. 50 µl of PCR reaction mix containing 100 ng of PRE TF 1 primer, 10X PCR buffer, and 2.5U of Taq (Mobio Laboratories, CA) was added to each RT reaction tube. PCR amplification was performed as described above.

Results

Detection of PERV gag sequences by PCR in tissue samples

Figure 1 shows representative PCR test results of PERV gag DNA in porcine tissue samples. DNA extracted from tissue samples was screened by PCR for the detection of gag gene sequences in 50 samples. In all the porcine tissue samples tested, gag sequences were ubiquitously present. Thus PERV proviral PCR assay detected the presence of PERV DNA in all the 50 porcine tissue samples tested.

Table 1 PCR analysis on porcine tissue samples for porcine endogenous retrovirus (PERV)

Method	Source	Sample load	Result	
			Positive	Negative
DNA-PCR	Porcine tissues	50	50	NIL
RT-PCR	Porcine tissues	25	20	5

RT: reverse transcriptase, PCR: polymerase chain reaction

Detection of PERV gag RNA sequences by RT-PCR in tissue samples

Figure 2 shows representative RT-PCR test results in PERV positive culture supernatants PK-15 PERV producer cell line and from porcine tissue samples. The RT-PCR assay detected the presence of PERV RNA in 20 of 25 porcine tissue samples.

Discussion

Laboratory surveillance of PERV in pig xenograft tissues is critical for determining the safety of porcine xenotransplantation [9]. We describe here a PCR-based assay for the molecular detection of PERV DNA and RNA sequences. Porcine xenografts are under evaluation for use in the treatment of a variety of life-threatening and chronic diseases. Pig genomes contain endogenous retroviral sequences encoding infectious type C retroviral particles. Initial reports suggested the host range of PERV to be restricted to porcine origin, but in 1997 Type C retrovirus from PK-15, termed PERV-PK was reported to infect human, mink and porcine cell lines. Because of the shortage of procuring human tissues, PERV found in the 1970s was checked for its safety in xenotransplantation and new treatments was evolved based on porcine tissues or cells, as *in vitro* infection of human cells lines, such as HEK-293, was demonstrated in 1997 [5].

The described PCR-based assays can be used to screen porcine tissue for PERV sequences. The PERV assays were designed with conserved PERV oligomers to allow the detection of all known PERV variants. We have developed PCR assays to detect proviral PERV gag sequences by using specific primers. All these PCR assays gave positive results for proviral DNA on porcine tissues. Since proviral DNA load does not necessarily correlate with the viral RNA load, we investigated whether the intact proviruses were dormant or biologically active. RT-PCR reaction was sufficient to detect constitutive transcription of PERV genomes in all tissues tested. Studies that have examined PERV RNA expression have detected constitutive transcription in many different organs including the kidney, heart, spleen, liver, thymus, lymph nodes and lung [10]. Our method included DNase pretreatment of RNA extracts, which was necessary to remove any residual PERV DNA that may originate from porcine cells. In addition, we included a control PCR reaction without RT to confirm that the positive RT PCR results were due to PERV RNA alone. Negative test results suggest the inability of the provirus to express functional viral RNA or the low titres of PERV RNA particles. The regulatory

Fig. 1 Detection of PERV proviral DNA from porcine tissues: Lane M represents the molecular marker (8–300 bp). Lane N represents the negative control of human peripheral blood lymphocyte (PBL). Lane P represents the positive DNA control from porcine PK-15 cell lysates (cell line in which PERV is released). Lanes 1 to 5 represent the porcine tissue samples positive for PERV.

Fig. 2 RT-PCR analysis of porcine tissue samples for PERV RNA gag sequences. Lane M represents the molecular marker (8–300 bp). Lane P represents the positive DNA control from porcine PK-15 cell lysates. Lane N represents the RT-PCR result in the absence of reverse transcriptase enzyme. Lanes 1, 2 and 4 represent the porcine tissue samples positive for PERV RNA. Lane 3 represents the tissue sample negative for PERV RNA by RT-PCR.

signals required for retroviral transcription, replication and integration into the host cellular DNA is located within the PERV long terminal repeat (LTR) sequence. Sequence analysis showed that most PERV proviral DNA was significantly mutated, thus suggesting the inability to express functional viral RNA [11].

Although the PCR assay described here was designed to detect all currently known PERV variants, the combination of these molecular assays with the detection of anti-PERV antibodies using PERV-specific serologic assays will provide additional data for the diagnosis of xenogenic

PERV infection [12]. Other molecular assays, such as generic detection of RT in plasma or culture recipient cells using ultra sensitive PCR-based RT assays, may be helpful in the detection of novel or variant pig retroviruses that may be transmitted from porcine xenografts [13–15].

The application of these PCR assays to porcine xenograft will help define the risks of PERV transmission to humans. The anticipated long-term survival of xenotransplant recipients could facilitate the adaptation of PERV to new human host or once transferred to human cells, PERV could conceivably recombine with related C-type human endogenous retrovirus (HERV) genomes. Recombination between PERV and HERV sequences could result in a new combinations of functional genes, potentially producing new replicating competent viruses. Although no data has indicated that PERV infection had occurred in any of the patients treated with porcine tissues or cells, as this virus belong to Retroviridae family, the worry is that it could infect humans and mutate as HIV did from chimpanzees. Thus, it is important that all tissues should be checked for PERV DNA/RNA prior to implantation of porcine xenografts to prevent the risk of retroviral infection.

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