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Quantitative Estimation of Porcine Endogenous Retrovirus Release from PK15 Cells

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A b s t r a c t

The present study focuses on the assessment of porcine endogenous retrovirus (PERV) release from PK15 cells in a time dependent manner. The highest amount of PERV A RNA was detected in PK15 cells after 16 hours of culture. The highest amount of PERV B RNA was detected in PK15 cells after 20 hours. The highest amount of both subtypes RNAs was detected in culture medium after 32 hours of culture. The peaks of PERV reverse transcriptase (RT) activity were detected after 28 h of culture in PK15 cells and after 32 hours in the culture medium. The monitoring of PERV release from PK15 cell line may be useful for the evaluation of PERV replication.

K e y w o r d s: dynamics, PERVs, PK15 cell culture, real time QRT-PCR

Transplantation has been proved to be in many cases very successful treatment, however limited availability of appropriate organs and tissues induces the search for alternative sources (Lechler *et al.*, 2005, Salim *et al.*, 2006; Fiorelli *et al.*, 2011). Pigs appear to be the most suitable animals that can be used as donors of cells, tissues and organs for human recipients (Jasinski *et al.*, 2006; Cozzi *et al.*, 2008), but immunological and biological barriers constitute the main obstacles of inter-species transplantation (Cozzi *et al.*, 2008).

The prevalence of porcine endogenous retroviruses (PERVs), which are permanently integrated in the genome of all pigs, involves the risk of a zoonotic viral infection (Scobie and Takeuchi, 2009). A prolonged contact of the human body with the animal tissue infected with the pathogens, and the use of immuno-suppressive drugs after grafting may facilitate pathogen adaptation and transmission to transplant recipients. PERVs can be released as infectious particles from different pig cells (McIntyre *et al.*, 2003; Cunningham *et al.*, 2004), and their infectivity is tightly connected with the dynamics of endogenous retroviruses production from host cells. Normal epithelial porcine kidney cells (PK15 cell line) seem to be an easily available model to study PERV release. The demonstration of the PERV dynamics may provide guidelines for the development of novel treatment strategies for potential PERV infection. Therefore, the present study focuses on the assessment of PERV release from PK15 cells in a time dependent manner. The quantitative monitoring of PERVs may be also useful for the evaluation of their replication activity.

A PK15 cell line (ATCC CCL-33) was obtained from the American Type Culture Collection and routinely maintained in the DMEM medium (Dulbecco's Modified Eagle Medium; Lonza, Basel, Switzerland), supplemented with 10% fetal bovine serum (FBS; Lonza, Basel, Switzerland) and gentamicin 25 mg/100 ml (Lonza, Basel, Switzerland). PK15 cells were seeded into 12-well culture plates (Nunc, Wiesbaden, Germany) at a density of 1×10^4 cells per well. Medium and cells were collected at the start of the experiment (0h) and after 4, 8, 10, 12, 16, 20, 24, 28, 30, 32, 34, 36, 38 and 40 hours. The cell-free supernatants were obtained by filtration through 0.45-mm-pore-size membranes.

Genomic DNA extraction was performed with a commercially available kit QIAamp DNA Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. Total RNA was extracted from harvested cells and the medium using the phenol-chloroform method (Chomczynski and Sacchi, 1987). The quality of isolated nucleic acids was checked using 0.9% agarose gel stained with ethidium bromide (Sigma-Aldrich, St. Louis, MO). Nucleic acids concentration was determined using GeneQuant II RNA/DNA spectrophotometer (Pharmacia Biotech, Cambridge, UK).

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The respective copy numbers of PERV A, PERV B DNA and PERV A, PERV B RNA were evaluated using real-time Q-PCR and SYBR Green I chemistry (SYBR Green QuantiTect PCR Kit, QIAGEN, Valencia, CA) and real-time QRT-PCR and SYBR Green I chemistry (SYBR Green QuantiTect RT-PCR Kit, Qiagen, Valencia, CA), respectively. The analyses were performed using an Opticon™ DNA Engine Continuous Fluorescence Detector (MJ Research, Watertown, MA). All samples were assayed in triplicate. Since the detection of PERV sequences in the culture medium could depend on the presence of residual pig cells, specific detection of the porcine cellular *GAPDH* gene and porcine mitochondrial DNA (mtDNA) was carried out. *GAPDH* was also included as an endogenous positive control of amplification and integrity of extracts. The following sequences of oligonucleotide primers, which are specific for the *env* gene of a particular subtype of PERVs and were described previously by Bösch *et al.* (2000), were applied: <u>PERV A</u> 5'-GAGATGGAAAGA-TTGGCAACAGCG-3' (sense), 5'-AGTGATGTTAGG-CTCAGTGGGGAC-3' (antisense); <u>PERV B</u> 5'-AAT-TCTCCTTTGTCAATTCCGGCCC-3' (sense), 5'-CCA-GTACTTTATCGGGTCCCACTG-3' (antisense). Detection of the porcine *GAPDH* gene was performed according Machnik *et al.* (2010), and the following sequences of oligonucleotide primers were applied: 5'-TGTCGCCATCAATGACCCC-3' (sense), 5'-TGAC-AAGCTTCCCATTCTC-3' (antisense), and porcine mtDNA was detected according to Moon *et al.* (2010), with primers: 5'-CTGAGGAGCTACGGTCATCAC-AAIIIIICTATCAGCT-3' (sense), 5'-TAGGGTTGTT-GGATCCGGTTTCIIIIIGGAATAGGA-3' (antisense) (I – inosine bases).

The thermal profile for Q-PCR was as follows: polymerase activation at 95°C for 15 min and then 45 cycles consisting of the following temperatures and time intervals: 94°C for 30 s, 65°C for 45 s and 72°C for 40 s. The thermal profile for one-step QRT-PCR was as follows: reverse transcription at 50°C for 30 min, polymerase activation at 95°C for 15 min and then 30 cycles consisting of the following temperatures and time intervals: 94°C for 30 s, 65°C for 45 s and 72°C for 40 s.

Specificity of PCR reaction and absence of the primer dimers were confirmed by determing the char-

acteristic melting temperature (Tm) of each amplimer. The PCR products were also separated on 6% polyacrylamide gel and visualized by silver staining.

Reverse transcriptase activity in the supernatant and harvested cells at the start of the experiment (0 h) and after 4, 8, 10, 12, 16, 20, 24, 28, 32 hours, respectively, was measured using a commercial assay (Cavidi-Tech, Uppsala, Sweden) according to the manufacturer's instructions.

Statistical analyses were performed using Statistica v 9.0 PL software (StatSoft, Tulsa, Oklahoma). P values below 0.05 were considered statistically significant. Values were expressed as median (Me) with the 25th and 75th quartiles. Non-parametric tests were used for statistical analyses because the Shapiro-Wilk test indicated that the data were not normally distributed. The Mann-Whitney U test was used to compare the copy number of PERV DNA in PK15 cells. The Friedman and the Wilcoxon tests were applied to assess differences in the copy number of PERV in PK15 cells and the medium during the culture.

Analysis revealed that the copy number of PERV A DNA was significantly higher than PERV B DNA in PK15 cells. The copy number of PERV A RNA was also significantly higher than that of PERV B in tested cells (Table I). In the culture medium, a statistically significant relation between viral DNA of PERV A and PERV B was not found. However, a significant difference was observed between viral RNA of PERV A and PERV B (Table I). All samples of the culture medium were found to be negative for the porcine *GAPDH* DNA and mtDNA, thus the possibility of contamination with residual porcine cells was excluded.

At the next step, time dependent changes in the RNA level of PERVs in the PK15 cell line were assessed. Statistically significant alterations of the copy numbers of PERV A (p = 0.00012, Friedman test) and PERV B RNA (p = 0.00012, Friedman test) were observed. However, the differences in the copy number of PERV A (p > 0.05, Wilcoxon test) and PERV B RNA (p > 0.05, Wilcoxon test) between the subsequent time points were not statistically significant. There was observed a stable release of PERV particles over a 40 hour period. The highest number of viral RNA of PERV A and PERV B in PK15 cells was detected after 16 and 20 hours, respectively

Table I

The quantitative relations between the copy number of PERV DNA and PERV RNA in the PK15 cell line and in the culture medium

	PK15 cells (copy number/1000 cells)			Culture medium (copy number/ml medium)		
	PERV A	PERV B	p*	PERV A	PERV B	p*
DNA	22904 (16216–31942)	> 7383 (5968–11456)	p<0.0001	1366 (530–2599)	> 863 (398–1582)	NS
RNA	1059974 (439580–2462898)	>443175 (248490–787153)	p=0.028	12964292 (7697625–20767705)	> 4839133 (2490295–10812063)	p=0.005

Statistical significance: p* < 0.05; p* – Mann-Whitney U test; NS – not significant
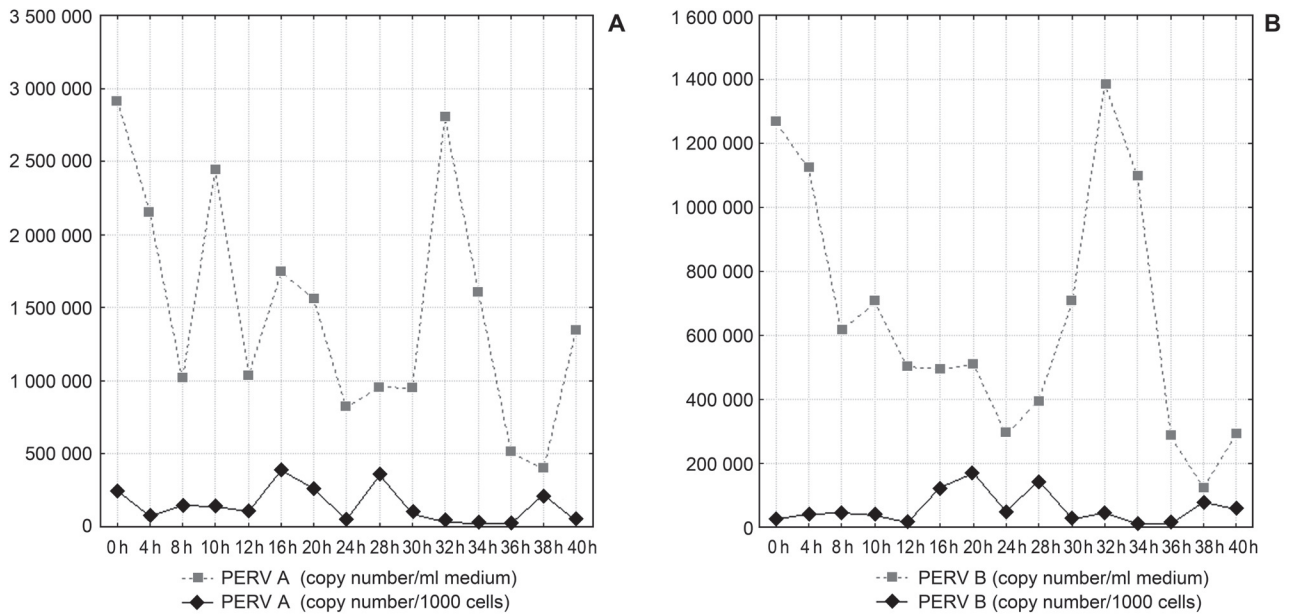Median with the 25th and 75th quartiles are presented

Fig. 1. The copy number of PERV A RNA (A) and PERV B RNA (B) in PK15 cells and the medium in the successive hours of culturing

(Figure 1A, Figure 1B, respectively). These changes in the level of virus RNA mirrored the alterations of the virus RNA in the culture medium. For the medium derived from the PK15 cells culture, the relations between the copy numbers of PERV A (p = 0.00012, Friedman test) and PERV B RNA (p = 0.00012, Friedman test) during all analyzed time points were similar. The highest increase in viral RNA of PERVs was noticed after 32 hours (Figs 1A, 1B).

During the next part of the study, the reverse transcriptase (RT) activity in PK15 cells and a cell culture supernatant was determined. We assayed RT activity from samples collected at the same time point as samples used for PERV RNA quantitative assays. Time dependent changes of RT activity in PK15 cells, as well as in the culture medium, were detected, with the peaks of PERV A and B RT activity in PK15 cells after 28 and 32 hours of culture, respectively (Fig. 2).

Since there are not enough human organs for allogeneic grafts, we need to look for alternative sources of organs. However, the endogenous retroviruses, permanently embedded in the genome of pigs, may be extremely important in this respect. They are not pathogenic for a host organism, but the role of PERVs in the pathogenesis of human diseases has been unknown so far. Moreover, until now the risk of PERV transmission to human cells *in vivo* is still unclear (Garkavenko *et al.,* 2004, Heneine *et al.*, 2001; Levy *et al.*, 2007, Switzer *et al.*, 2001; Valdes-Gonzalez *et al.*, 2010).

The presence of PERVs in various porcine cells and tissues was demonstrated by the authors of most research, for instance Armstrong *et al.* (1971) using electron microscopy detected gammaretroviruses in the porcine kidney cell lines: PS, PK15, IB-RS-2 and SK6O.

Similarly, Suzuka *et al.* (1985; 1986) identified C-type virus particles or unintegrated closed circular DNA of porcine retrovirus in swine cell line. Sypniewski *et al.* (2005) and Tacke *et al.* (2003) showed the presence of PERVs in porcine peripheral blood mononuclear cells (PBMCs) and fragments of the liver, kidney, and heart. A similar study was conducted by Prabha and Verghese (2009), authors demonstrated that PCR is a rapid and specific test for confirmation of the presence of PERVs. However, the most of these authors demonstrated only the presence of porcine endogenous retroviruses in
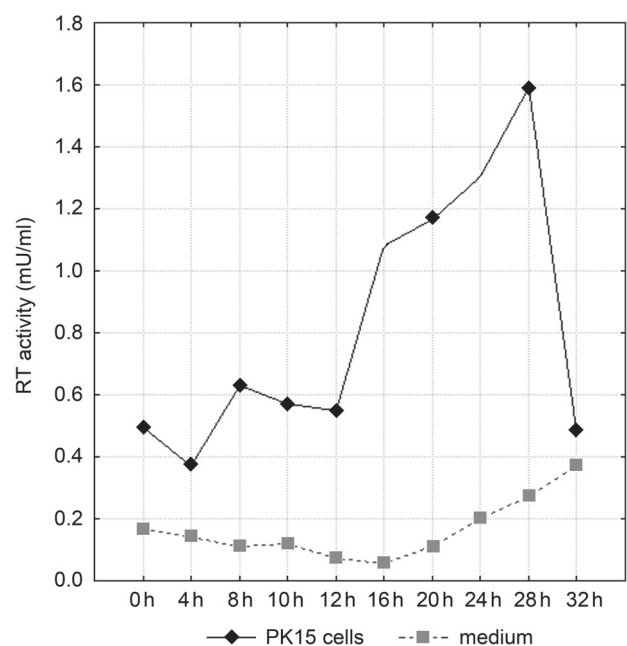

Fig. 2. The changes of PERV RT activity in PK15 cells and the culture medium in the successive hours of culturing

examined tissues without quantitative assessment of PERV subtypes.

Our results revealed that the copy number of PERV A DNA and PERV A RNA was significantly higher than that of PERV B DNA and PERV B RNA in PK15 cells. Sypniewski *et al.* (2005), tested only DNA levels in porcine tissues and did not indicate significant differences in the copy number of PERV A and PERV B DNA. These authors showed that the copy number of another virus variant – PERV C DNA, is the lowest when compared with the level of A and B subtypes. Absence of PERV C in a PK15 cell line we used in our experiments (Ferrari *et al.*, 2003) and in some porcine tissues can help to select organs for transplant. Elimination of tissues with PERV C can be beneficial for transplantation safety because the emergence of more infectious recombinant PERV A/C is limited (Bartosch *et al.*, 2004; Harrison *et al.*, 2004).

Cellular PERV RNA expression does not necessarily imply the release of viral particles; therefore RNA should also be prepared from cell culture supernatants. Like other authors (Tacke *et al.*, 2003; Cunningham *et al.*, 2004), we observed viral RNA both in the PK15 cell line and in the culture medium. It can be assumed that retroviruses integrated in the PK15 genome are capable to complete their life cycles and have the potential of causing infection. Kim *et al.* (2009) reported that PERVs produced by PK15 cells were able to infect human cells.

In our study, we observed a stable release of PERV particles over a period of 40 hours. We also found that the copy number of PERV A and B RNA in the culture medium fluctuated in a time dependent manner. The increase of PERV RNA in the supernatant may be caused by the release of virions from PK15 cells. At the time we initiated our study, only few published research reported the release of porcine endogenous retroviruses from porcine cells in time dependent fashion. Similarly to our results, Kim *et al.* (2009a) showed the stationary production of the PERV in host system. The studies performed by Tacke *et al.* (2003) and Cunningham *et al.* (2004) revealed that stimulation of porcine cells induced PERV release. Cheung and Bolin (2002) determined the kinetics of PCV2 (porcine circovirus type 2) replication in PK15 cells. These authors showed that the biosynthesis of viral antigens, RNA transcripts and progeny viruses increased in a time dependent manner during virus replication (Cheung and Bolin, 2002).

Further verification of PERV virion production was achieved by the detection of RT activity as a retroviral marker both in PK15 cells and cell culture supernatants. In our study, RT activity was detected in all tested PK15 cell samples and the supernatants of cultured cells. It was observed that RT activity fluctuated in a time dependent manner, corresponding with the changes of the copy number of PERV RNA. In the previous research, it was shown that the liver and muscle cell-free supernatants and the serum samples of pigs demonstrated high PERV RT activity, which implicated that PERVs were actively replicating (Liu *et al.*, 2005). Only Tacke *et al.* (2003) and Han *et al.* (2011) determined the release of RT activity into the culture supernatant of porcine stimulated PBMCs and porcine hepatocytes, respectively, at different time intervals of culturing. The latter authors demonstrated that the RT activity was the highest after 10 hours and 2 days of culturing.

Our study on viral dynamics provides a timeline of PERVs release, which may be useful for potential preventive or antiviral strategies in xenotransplant recipients. Unfortunately, there is almost unexisting data on the life cycle of PERVs.

Summarizing, the copy numbers of PERV A and PERV B RNA, both in PK15 cells and in the culture medium, slightly changed in a time dependent manner. The monitoring of PERV release from the PK15 cell line may be useful for the characteristics of PERVs replication. Another important aspect of PERV production is assessment of its influence on the infection of human cells. Further study is required to define the pathogenic influence of porcine endogenous retroviruses on human cells. Such study can be used as a foundation to develop neutralization assays and antigen preparation for immunological testing of infections. Elimination of risks will enable the introduction of interspecies transplants to medicine.

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