

Comparison of the age-related porcine endogenous retrovirus (PERV) expression using duplex RT-PCR

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Porcine endogenous retroviruses (PERVs) are members of family *Retroviridae*, genus *Gamma retrovirus*, and transmitted by both horizontally and vertically like other endogenous retroviruses (ERVs). PERV was initially described in the 1970s having inserted its gene in the host genome of different pig breeds, and three classes, PERV-A, PERV-B, and PERV-C are known. The therapeutic use of living cells, tissues, and organs from animals called xenotransplantation might relieve the limited supply of allografts in the treatment of organ dysfunction. Because of ethical considerations, compatible organ sizes, and physiology, the pig has been regarded as an alternative source for xenotransplantation. Sensitive duplex reverse transcription-polymerase chain reaction protocols for simultaneously detecting PERV *gag* mRNA and porcine glyceraldehydes 3-phosphate dehydrogenase mRNA in one tube was established. To compare the age-related PERV expression patterns of the lung, liver, spleen, kidney, heart, and pancreas in commercial pigs, 20 pigs from four age groups (5 heads each in 10 days-, 40 days-, 70 days-, and 110 days-old, respectively) were used in this study. The expression patterns of PERV were statistically different among age groups in lung, liver, and kidney (ANOVA, $p < 0.05$). These data may support in the selection of appropriate donor pigs expressing low levels of PERV mRNA.

Keywords: mRNA expression, PERV, pig, RT-PCR

Introduction

Endogenous retroviruses (ERVs) are integrated into the germ-line of vertebrates and transmitted to their offspring by Mendelian genetics. The genomic structures of ERVs include group specific antigen (*gag*), polymerase (*pol*),

and envelope (*env*) genes which are flanked with 5' and 3' long terminal repeat (LTR) possessing regulatory elements [15]. The expression of ERV genes and their adjacent genes is controlled by transcription regulatory elements placed on the LTR [13]. The majority of ERVs are transcriptionally inactive because deletions and point mutations interrupt the coding potential of the *gag*, *pol*, and *env* genes [14].

The pig also harbors endogenous retroviruses, called porcine ERV (PERV). The first report on PERV was reported in the 1970 [4]. PERVs are the members of family *Retroviridae*, genus *Gamma retrovirus* and vertically transmitted like other ERVs [19]. PERV inserted its gene in the pig genome in approximately 30 to 50 sites [1] and 3 classes, PERV-A, PERV-B, and PERV-C, are known [17]. These classes display high sequence similarity in the genes coding for the *gag* and the *pol* but differ in the genes encoding the *env* proteins [21].

In xenotransplantation research, every cell or tissue from a porcine xenograft was thought to carry PERV and could act as a potential source of retrovirus, which could not be eliminated by keeping pigs under specific-pathogen-free conditions or by simple outcross-breeding protocols. Fortunately, there were no trans-species infections of PERV in many *in vivo* porcine cell or organ transplantation trials [11]. However, PERV-A and -B could successfully infect human originated cell lines *in vitro* [9,21]. In addition, the state of immunosuppressed patients cannot be excluded in xenotransplantation. Recently, an *in vivo* study of PERV infection into human cells in a nude mouse suggests the possibility of indirect human PERV infections [25].

In previous studies, many techniques including conventional polymerase chain reaction (PCR), reverse transcription (RT)-PCR [3,16,20], real time PCR, real time RT-PCR [2], and monoclonal antibodies [5,10] were developed to analyze the risk of PERV transmission. Comparisons of PERV mRNA expression patterns were

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Table 1. Primers for porcine endogenous retrovirus (PERV) group specific antigen (*gag*) gene and porcine glyceraldehydes 3-phosphate dehydrogenase (GAPDH)

Target	Direction	Sequences (5'→3')	Size
Pig GAPDH	Forward	CGTCAAGCTCATTTCCTGGTACG	220 bp
	Reverse	GGGGTCTGGGATGGAAACTGGAAG	
PERV <i>gag</i>	Forward	TCAGGCGGTACACCCCTTT	150 bp
	Reverse	GATCACGTAACCTCAGCCTCCTGTAA	

Sequences were referred in the Genebank information of pig GAPDH (accession No. X94251) and PERV *gag* (AF038600).

conducted to determine the viral load in various porcine tissues. In that study, the kidney showed the highest expression levels and the pancreas showed the lowest. The assessment of viral load could potentially reduce the risk of PERV transmission [8], and help select appropriate donor pigs expressing low levels of PERV mRNA.

The objective of this study was to establish sensitive duplex RT-PCR protocols for detecting PERV *gag* mRNA and porcine glyceraldehydes 3-phosphate dehydrogenase (GAPDH) mRNA. In addition, this technique was used to compare the age-related expression levels of various tissues in commercial pigs.

Materials and Methods

Pigs

Twenty pure breed Duroc pigs were allocated into 4 different age (10, 40, 70, 110 days) groups. All animal experiments were in compliance with the current laws of Korea. Care and treatment of animals were conducted in accordance with the protocols and guidelines of the Seoul National University Institutional Animal Care and Use Committee, Korea.

RNA extraction

The organs used for this study were the lung, liver, spleen, kidney, heart, and pancreas. Each organ from a pig was collected and picked up 0.1 g of piece, separately. Collected tissue was minced and suspended in 1 mL of Dulbecco's modified Eagle media without fetal bovine serum. RNA was extracted using TRIzol (Invitrogen, USA) according to manufacturer's recommendations. Briefly, 250 μ L of the homogenated samples was mixed with 750 μ L of TRIzol and incubated for 15 min at room temperature. Following the addition of 200 μ L of chloroform, the mixture was centrifuged at 12,000 \times g at 4°C for 15 min. After adding an equivalent volume of 2-propanol to the supernatants for RNA precipitation followed by 15 min incubation at room temperature, further centrifugation was performed at 12,000 \times g at 4°C for 10 min. RNA pellets were washed with 1 mL of 75% ethanol, and centrifuged at 12,000 \times g at 4°C for 5 min.

Pellets were resuspended in 30 μ L of diethylpyrocarbonate (DEPC)-treated deionized water after drying.

Primers

Primers for duplex PCR with PERV *gag* and porcine GAPDH were employed as previously designed [16,24]. The sequences of primer sets are listed in Table 1.

RT and PCR

Prepared RNA was treated with DNase (Promega, USA) for 30 min at 37°C according to manufacturer's protocol. Reverse transcription was performed using a random hexamer primer (TaKaRa, Japan) and M-MLV reverse transcriptase (Invitrogen, USA). The random primer (100 pmol) and 1 μ g of DNase-treated RNA were mixed, heated at 95°C for 5 min, and then immediately chilled on ice. The remaining reagents, including \times 5 first strand buffer (50 mM Tris HCl, 75 mM KCl, 3 mM MgCl₂), 10 mM DTT, 0.3 mM each dNTP, and 100 units of reverse transcriptase, were added, making a final volume of 20 μ L. The mixture was incubated at 37°C for 1 h.

Amplification was performed using the GeneAmp PCR systems (Model 2700; Applied Biosystems, USA). For PERV *gag* RNA and pig GAPDH detection, 1 μ L of cDNA obtained by reverse transcription described above, 0.5 μ M of each primers and 16 μ L of *i*-StarMaster mix solution [0.25 mM each of dNTP mixture, 10 mM of Tris-HCl (pH 9.0), 2 mM Mg²⁺ solution and \times 1 chemical stabilizer II] were mixed and adjusted to 20 μ L with DEPC treated DW using *i*-StarMaster mix PCR kit (iNtRon Biotechnology, Korea) containing 2.5 units of *i*-Star*Taq* DNA polymerase and \times 1 chemical stabilizer I and \times 1 loading buffer.

The amplification procedures were as follows: 35 thermal cycles consisting of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Upon completion of the cycles, samples were maintained at 72°C for 5 min, prior to cooling. PCR products were analyzed by electrophoresis on a 2.0% agarose gel containing ethidium bromide. Loading was executed three times with 5 μ L of PCR products in order to obtain statistical analyses of the band densities. Pictures were taken of each electrophoresis using Gel Doc XR

(Bio-Rad Laboratories, USA).

Sensitivity test

To determine the sensitivity of duplex RT-PCR, RNA was extracted from PK-15 cells, and treated with DNase (Promega, USA) according to manufacturer's guides. DNase-treated RNA concentrations were measured with a spectrophotometer (Eppendorff, Germany) at 260 nm. RNA was measured as 60 ng, and serially diluted to a concentration of 6 pg, followed by reverse transcription. The corresponding cDNA samples were used to amplify *gag*, and pig GAPDH.

Density analysis

In order to confirm that there were no differences in the expression ratio between duplex RT-PCR and separate tube RT-PCR with each primer set, the two methodologies were compared. Reactions were conducted in three categories: one containing only PERV *gag* primer, another containing only pig GAPDH, and the last mixing both PERV *gag* and pig GAPDH together with cDNA from the PK-15 cell. The ratio between the separate reaction and duplex reaction of RT-PCR was compared.

Image of the gel electrophoresis was obtained and the densities of the amplified DNA bands were measured with a Quantity One quantitation analysis software package (Gel Doc XR; Bio-Rad Laboratories, USA) according to the user manuals. The ratio of the PERV *gag* / pig GAPDH was expressed so the density of the lower band was divided by the density of upper band which represents PERV *gag* and pig GAPDH, respectively. The mean ratio of the individual pigs was calculated using the density from three pictures which were from three different duplex RT-PCRs from the same sample. Individual pig expression ratios were compared with different ages to investigate the relationship between expression levels and ages using ANOVA with Tukey test.

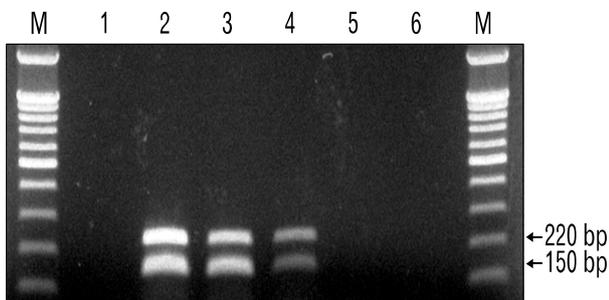


Fig. 1. Sensitivity of the duplex RT-PCR detecting PERV *gag* RNA and porcine GAPDH mRNA in PK-15 cell. PERV *gag* was detected in 150 bp and pig GAPDH detected in 220 bp. Lane M: 100 bp DNA ladder, Lane 1: DW, cDNA from Lane 2: 620 ng, Lane 3: 6.2 ng, Lane 4: 620 pg, Lane 5: 62 pg, Lane 6: 6.2 pg of mRNA concentration.

Results

Sensitivity of the developed duplex RT-PCR

The detection limit of the designed duplex RT-PCR was 620 pg for both of PERV *gag* and pig GAPDH from the PK-15 cell RNA (Fig. 1). The expected product could be successfully distinguished on agarose gels.

Comparison of the PERV *gag* and pig GAPDH RNA expression

The mean expression levels from separate tube RT-PCR and single tube duplex RT-PCR were 0.559 and 0.561, respectively, with no statistical difference between the two reactions ($p > 0.05$).

The standard deviations of the expression ratio in each pig after three trials of RT-PCR were between 0.01 and 0.16 in all of the tested pigs. This was presented together with the mean expression ratio for each pig in a bar chart.

As shown in Fig. 2, established duplex RT-PCR could differentiate two expected products from pig tissues. However, there were no successful amplifications in any pancreas tissue samples. The density of DNA bands which were displayed was measured and the PERV *gag* / pig GAPDH ratio was calculated, and these data grouped vertical bars plot (Fig. 3). The PERV expression patterns were statistically different among each group in lung, liver, and kidney ($p < 0.05$). However, there was no statistical difference among each group in the spleen and heart. In the lung and kidney, the PERV *gag* expression level of the 110 day group was statistically lower than the 10 and 40 days groups, and differences between 40 and 70 day groups were not statistically significant ($p < 0.05$). In the liver, the 40 day group was significantly lower than the 10 and 70 day groups ($p < 0.05$).

Discussion

A sensitive duplex RT-PCR protocol, which could detect PERV *gag* RNA and pig GAPDH mRNA simultaneously, was established in this study. This technique enabled the simultaneous comparison of the PERV *gag* mRNA and pig GAPDH mRNA expression levels using multiplex RT-PCR. The single tube duplex RT-PCR might be the reasonable for this research, as no differences in PERV *gag* to pig GAPDH ratio was shown in comparison between separate tube RT-PCR each tube containing only one set of primers for PERV *gag* or pig GAPDH and single tube duplex RT-PCR. The expression ratio of the PERV *gag* and GAPDH was not age-related, but the patterns of expression levels in each age were different with each organ. A previous study [8] reported that the PERV mRNA expression level was highest in the kidney among the various tissues tested, and lowest in the pancreas. In

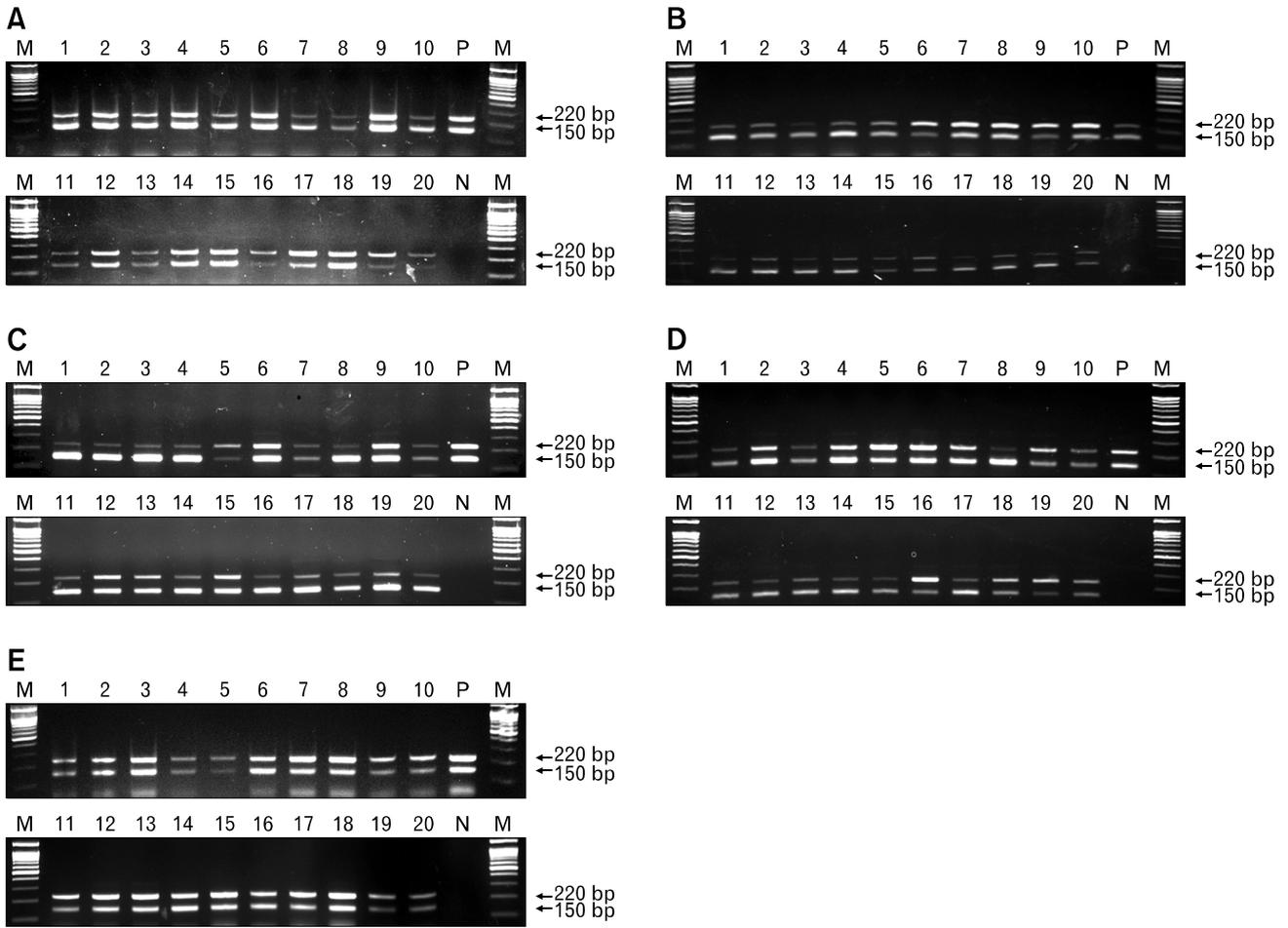


Fig. 2. Duplex RT-PCR on the commercial pig detecting PERV *gag* and pig GAPDH mRNA in lung (A), liver (B), spleen (C), kidney (D), and heart (E). Lane M: 100 bp DNA ladder, Lane P: PK15 cell, Lane N: DW, Lane 1-5: 10 days-old group, Lane 6-10: 40 days-old group, Lane 11-15: 70 days-old group, Lane 16-20: 110 days-old group.

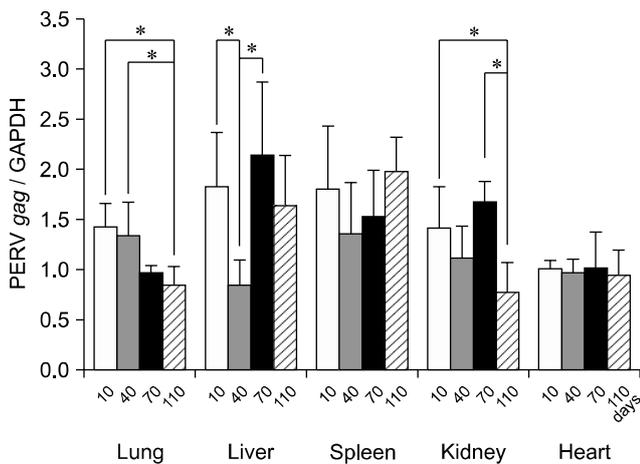


Fig. 3. The columns represent mean expression ratio (PERV *gag*/GAPDH) of each age group and standard deviations. The results of all the tested organs were presented same plot. *Statistical differences between age groups ($p < 0.05$).

in addition, more detailed comparisons were performed according to pig breeds or between pig organs. Age-related PERV mRNA expression patterns in the kidney were similar with previous studies [8,23] on retroviral loads in viremia. The viral titer of the pigs was the highest at 2 to 6 months and lowest at 6 months [23]. Moreover, pigs in the low health status showed elevated levels of viremia compared to those of high health status [23].

Compared to a previous study, this study was conducted in four different age groups, 10, 40, 70, and 110 days-old, which could represent the stage of suckling, nursery, grower, and finisher, respectively. In a similar study performed by Tucker *et al.* [23], age groups were classified into three groups, < 2 months-old, 2 to 6 months-old, and > 6 months-old and then the endpoint of age was above 6 months-old. This classification included various age stages of corresponding groups. However, animals of the same age were included in this study with the endpoint at 110 days-old, which was less than 4 months.

The increasing and decreasing tendencies of PERV between the two studies were similar in the kidney, but the timing of dropping and the patterns in other organs were different. Furthermore, the patterns of PERV mRNA expression with respect to age varied in the organs. In spleen and heart, there was no significant difference in expression levels. The differences among the each age group were observed in the lung, liver, and kidney. However, the patterns of PERV mRNA according to age were dissimilar among the three organs. It was difficult to determine the reason behind the differences. The physical and/or physiological or *environmental* differences might be one of the reasons. The regulatory signals for ERV were reported as cell and tissue types, and processes were related to differentiation and aging, cytokines and steroids [22]. In addition, various stress signals including injury, infection, oxidative stresses and psychological stresses could modulate their transcription [7]. As a lot of factors can affect PERV expression, more elaborate experiments considering various controlled factors should be done to obtain more clarity on this subject.

Unfortunately, even though the pancreas was tested, there were no amplifications. This could be due to the large amount of ribonuclease A as well as other digestive enzymes in the pancreas that may have degraded RNA [6]. The errors in tissue transport and temperature variations during tissue handling may have affected the RNA in tissue samples. Also, the technical errors in the RNA extraction process or storage of tissue homogenate might accelerate the breakdown of RNA. Moreover, since the pancreas contains more ribonuclease than other tissues, it could be affected more by the inadequate treatment of samples.

To compare PERV expression levels, it is better to estimate the expression level of the all three genes, *gag*, *pol*, and *env*. Moreover, in envelope genes, the estimation of the *envA*, B, and C mRNA expression levels would be better. Unfortunately, only the *gag* gene was employed in the current study to investigate the expression of PERV regardless of subtypes because *gag* is highly conserved in PERV and the expression of *gag* gene is essential for virion production [18].

This study would be better if all the pigs bred in Korea were included. However, this research was focused on comparing PERV expression to age in pigs. For that reason, pig species was limited to Duroc. And in order to control for the species, the use of pure bred might be beneficial for research. The study about PERV expression in different pig species describing expression of envelope gene common mRNA and envelope A, B, and C was conducted in previously [12]. Though it is difficult to compare with this study because this study only dealt with *gag* mRNA, expression of envelope genes in previous study [12] presented various phages. Especially, *envA* and *envB* were not expressed in Duroc pig which was used in

current study.

Even though lymph nodes are good samples for PERV expression, only the major organs were tested in this study because the focus was on the organs which might be used for transplantations. In addition, the PERV expression in embryonic stages might be covered in further studies.

In conclusion, even though consistent age related patterns in expression of PERV mRNA was not observed, the comparison method using duplex RT-PCR with GAPDH was established to be effective in this study. Since elimination of PERV is nearly impossible, the best way might be to focus on reducing the risk of PERV transmission. Finding a suitable donor expressing a lower level of PERV mRNA than the others could diminish the potential risk of PERV transmission.

Even though the physiologic identification was important, the microbiologic safety should be regarded in the concept of public health. Furthermore, established techniques might be helpful in decreasing the infection risk.

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