Original Article
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Analysis of swine leukocyte antigen class I gene profiles and porcine endogenous retrovirus viremia level in a transgenic porcine herd inbred for xenotransplantation research

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Molecular characterization of swine leukocyte antigen (*SLA*) genes is important for elucidating the immune responses between swine-donor and human-recipient in xenotransplantation. Examination of associations between alleles of *SLA* class I genes, type of pig genetic modification, porcine endogenous retrovirus (PERV) viral titer, and PERV subtypes may shed light on the nature of xenograft acceptance or rejection and the safety of xenotransplantation. No significant difference in PERV *gag* RNA level between transgenic and non-transgenic pigs was noted; likewise, the type of applied transgene had no impact on PERV viremia. *SLA-1* gene profile type may correspond with PERV level in blood and thereby influence infectiveness. Screening of pigs should provide selection of animals with low PERV expression and exclusion of specimens with PERV-C in the genome due to possible recombination between A and C subtypes, which may lead to autoinfection. Presence of PERV-C integrated in the genome was detected in 31.25% of specimens, but statistically significant increased viremia in specimens with PERV-C was not observed. There is a need for multidirectional molecular characterization (*SLA* typing, viremia estimation, and PERV subtype screening) of animals intended for xenotransplantation research in the interest of xeno-recipient safety.

Keywords: MHC class II genes, *Retroviridae,* heterologous transplantation, infection, viremia

Introduction

Increased use of porcine organs in xenotransplantation is currently an important trend in preclinical studies, and such usage may overcome the growing shortage of human organ donors. Although pigs are preferred as xenogenic donors, immunological responses to their organs have occurred, thereby precluding application of xenografts in clinical situations. Immunological transplant barriers can be ruled out through the generation of transgenic pigs with knocked-out expression of genes encoding some important mediators of host-graft interactions [25,33,38]. However, such genetic manipulations may lead to unexpected side effects. One of the questions that remains unanswered is whether transgenic pigs have their major histocompatibility complex (MHC) protein expression profile unmodified. In pig-to-human xenotransplantation, human immunological cells (mostly CD8+ T lymphocytes and natural killer [NK] cells) directly recognize swine leukocyte antigen (*SLA*) class I molecules, which leads to graft destruction.

Hyperacute graft rejection, occurring within several minutes after transplantation, is a major problem in xenotransplantation [29]. This hyperacute response is associated with complement system activation by antibodies against the Gal antigen, which is present in glycoproteins and glycolipids on the cell surface. Therefore, there is a need to detect mechanisms that control the occurrence of xenograft rejection and devise methods to counteract such rejection. The most effective solutions to date are the creation of genetically modified pigs with permanent inactivation of the gene (α(1,3)galactosyltransferase, α*1,3GT*) responsible for Gal antigen synthesis [5,17] or the modification of donor cell surface proteins, including fucosyltransferase II (*FUT2*) or α-galactosidase (*GLA*) genes that code insertion to the donor genome. The FUT2 enzyme uses the same acceptor, N-acetyllactosamine, as α1,3GT, but it leads to formation of an

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H epitope and displaces Gal antigen synthesis, which significantly reduces immunogenicity [33]. As a consequence of α-*GLA* excising the terminal α-D-galactose unit from the Gal epitope, there is a tenfold decrease in binding capacity of antibodies specific for porcine Gal antigen; however, complete elimination of galactose units from the Gal epitope using *FUT2* transgenesis is not possible. Interestingly, an additive effect of double transfection (*GLA* and *FUT2* genes) has been observed, and it resulted in complete absence of the Gal epitope on the cell surface [25]. In addition, regulation of the complement system may influence positive on xenograft acceptance. One of the complement system blocking agents is factor CD59 (membrane inhibitor of reactive lysis) that prevents entry of the complement complex to the cell membrane and weakens the immune response against the transplanted organ.

The *SLA* gene complex is located within chromosome 7, spanning the centromere. This is one of the most gene-dense regions in the porcine genome, much more compact than the equivalent human MHC region, and it includes three major gene clusters: class I (1.1 Mbp), II (0.7 Mbp) (mapped to the chromosome short arm, band 7p1.1) (Fig. 1) and III (0.5 Mbp) (on the long arm, band 7q1.1). It is characterized by high polymorphism with 223 alleles of *SLA* class I being characterized by 2013 [12]. To avoid the implication that some loci are homologous to the genes of HLA system, *SLA* class I genes have been assigned successive numbers. It was suggested that the sequence and overall genomic organization of *SLA* class I genes was quite different from those in the region coding the human MHC [19].

Describing the mechanisms involved in immune system regulation is crucial for the long-term prospects of pig-to-human xenotransplantation. Special attention should be paid to xenograft acceptance or rejection and development of immune tolerance of recipients toward transplanted swine tissue. *SLA* class I proteins, present on the surface of virtually all nucleated porcine cells, are directly recognized by human CD8+ T cells and NK cells and can kill swine cells and cause transplant destruction [34]. Xenoantigens and interleukin 2 (IL-2) can stimulated CD8+ T cells, which leads to differentiation into highly reactive nonspecific cytotoxic cells. Such cells can destroy nonspecifically adjacent xenograft cells, but beyond the xenograft, their activity is almost completely reduced due to the absence of IL-2 in sufficiently high concentrations [36]. The binding site for the CD8 co-receptor is located on the α3 extracellular domain. Together with two others domains (α1 and α2), constituting transmembrane and cytoplasmic regions, α3 forms glycoprotein (45 kDa) bound to β_2-microglobulin. This protein is the product of constitutively expressed *SLA-1*, *SLA-2*, and *SLA-3* genes, while the remaining components of the *SLA* class I complex are pseudogenes. Based on sequencing analysis, a greater similarity was reported between *SLA-1* and *SLA-3* than between both of them and *SLA-2* [19,37].

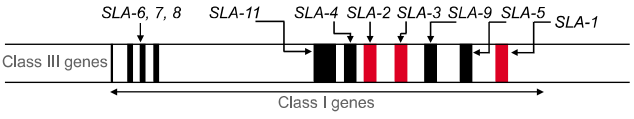

Fig. 1. Schematic organization of the *SLA* class I gene cluster including three major gene groups: class I (1.1 Mbp), class II (0.7 Mbp) (mapped to the chromosome short arm, band 7p1.1), and class III (0.5 Mbp) (on the long arm, band 7q1.1). *SLA-1*, *-2*, and *-3* genes are indicated in red. *SLA*, swine leukocyte antigen.

Using transgenic pigs as a source of cells or organs for xenotransplantation is associated with the risk of potentially dangerous zoonoses, including porcine endogenous retrovirus (PERV) transmission [20,21,24,42]. PERVs are integrated in many copies of the pig genome, present in all tissues, and are released from cells; however, their mechanism of infection in animals and humans remains undescribed. Transcriptionally silent, but potentially replicative competent proviruses, may activate and infect human cells. There are three identified classes of infectious type PERVs (PERV-A, PERV-B, and PERV-C) characterized by distinct *env* genes and highly homologous sequences in the rest of the genome [3]. The PERV-A and -B subtypes are present, at different copy numbers, in the genome of all pig strains, whereas PERV-C is present in many, but not all specimens. In addition, the C subtype has a narrow host range and infects only porcine cells, as opposed to the A and B subtypes, which are polytropic. The presence of PERV-C in the genome of xenograft cells is potentially risky because of possible recombination with other PERV subtypes [3,8,15]. Because PERVs are found in the DNA of all pigs, they cannot be eliminated from all xenotransplants [8]. However, PERV-C−positive pigs can be excluded from an organ donor cluster by undertaking preliminary PERV subtype identification. Another issue that needs closer examination is the potential effect of *SLA* gene expression on PERV replication and whether the viral load level is attributable to the characteristic *SLA* class I gene profile. Elucidation of the associations between alleles of *SLA* class I genes, genetic modification of pigs, and the viral titers and subtypes of PERVs may provide important information about xenograft acceptance or rejection and the safety of xenotransplantation.

Materials and Methods

Animals

Porcine (*Sus scrofa domestica*) peripheral blood samples, obtained from a herd of Polish Landrace pigs, maintained by the National Research Institute of Animal Production (Balice, Poland), were collected in EDTA solution. The herd included several types of genetic modifications: nineteen specimens were

modified by *FUT2* gene transfection, fifteen by human *GLA* gene, and four pigs by double transgenesis (*FUT2* and *GLA* genes). One animal had additional copies of the *CD59* gene, and 11 pigs remained unmodified, constituting the control group. Production of transgenic specimens had been performed by microinjection of exogenous DNA constructs into the male pronucleus of a fertilized egg cell [18]. The transgenic herd was obtained via Developmental Project N R12 0036 06/2009 (The National Centre for Research and Development, Poland) entitled: Obtaining of transgenic pigs as donors of tissue and organs for transplantation in humans and their biotechnological, physiological and medical characteristics (leader Prof. Ph.D. Z. Smorąg). This study was approved by the Local Ethics Committee for Animal Experiments, Institute of Pharmacology, Polish Academy of Sciences, Cracow (No. 601/2008).

SLA class I genes analysis

Total RNA was extracted with Tri-Reagent LS (Sigma-Aldrich, USA), according to manufacturer's recommendations. Cell line PK-15 (CCL-33; American Type Culture Collection [ATCC], USA) of porcine kidney origin was initially used as a standard template to optimize conditions of reverse transcription polymerase chain reaction (RT-PCR) for *SLA*-specific reactions. RT reactions were carried out with 1 μg of total RNA of each extract along with MMLV High Performance Reverse Transcriptase (Epicentre, USA) and oligo$(dT)_{15}$ primers (Novazym Polska, Poland). Subsequently, each cDNA sample served as a PCR template with nine separate primer sets: three for detection of *SLA-1* profile, two for *SLA-2*, and four for *SLA-3* (Table 1) [2]. The chosen primer sets allowed amplification of the 3′ parts of exons 1 and 2 and most of exon 3 in three *SLA* class I genes. The *SLA*-specific PCR reactions were conducted under optimized conditions with 1 U *Taq* DNA polymerase (Thermo Fisher Scientific, USA) in 15 μL volumes containing *Taq* buffer, 2 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.67 μM of each primer, and 1.5 μL of cDNA as a template. The PCR thermal profile used included an initial denaturation at 95°C for 3 min, 30 cycles of 95°C for 30 sec, 65°C for 30 sec, and 72°C for 30 sec, and final extension was performed at 72°C for 3 min. *SLA* class I profiles were designated based on 6% polyacrylamide electrophoretic separation of the obtained RT-PCR and *SLA*-specific products, with post-staining in a solution of 3× GelRed (Biotium, USA) in 1× TBE buffer followed by digitizing with an InGenius LHR system and GeneTools software (Synoptics, UK).

Viral load level estimation

Every specimen of the herd was analyzed to determine the PERV viral load in peripheral blood. For this purpose, a quantitative RT-PCR technique (real-time RT-PCR) using an Mx3000P Real-Time RT-PCR System (Stratagene, USA) was applied. Total RNA extract (100 ng) from peripheral blood was used as a template. The general level of PERV particles was determined on the basis of *gag* RNA copies with the following primers: forward 5′-TGA TCT AGT GAG AGA GGC AGA G-3′ and reverse 5′-CGC ACA CTG GTC CTT GTC G-3′ (amplification product, 262 bp) [26] in a final 0.2 μM concentration. The mRNA of a porcine *gapdh* gene fragment, constituting an endogenous control of the reaction, was amplified with the use of primers previously described by Machnik *et al*. (Table 2) [21]. Brilliant II SYBR Green QRT-PCR Master Mix Kit (Stratagene) was applied to amplify the target sequences. One-stage real-time RT-PCR was conducted as follows: RT, 50°C for 30 min; initial denaturation, 95°C for 10 min (40 cycles of PCR [94°C for 30 sec, 61°C for 30 sec, and 72°C for 30 sec]); and a final 10 min elongation at 72°C. To confirm the amplification specificity, melting temperature of the amplimers was determined by initial incubation at 95°C for 1 min followed by lowering the temperature 55°C and then slowly increasing the temperature (0.2°C per sec) to 95°C with simultaneous fluorescence reading.

Detection of PERV subtypes within the herd

Genomic DNA from peripheral blood samples was isolated by using GenElute Blood Genomic DNA kits (Sigma-Aldrich). To detect subtypes of PERV present in the pig genome, amplification by multiplex PCR with *env*-specific primers [4] (Table 2) was conducted. Porcine *gapdh* fragment amplification

Table 1. Primers used for swine leukocyte antigen (*SLA*) class I genes analysis

Locus	Sequences	PCR product length (bp)
SLA-1a	F: 5′-CCTCTTCCTGCTGCTGTCG-3′ R: 5′-ACTCCACACACAGTCCCTGC-3′	548
SLA-1b	F: 5′-CCTCTTCCTGCTGCTGTCG-3′ R: 5′-CCTCCACACACCGGCCCTG-3′	548
SLA-1c	F: 5′-CCTCTTCCTGCTGCTGTCG-3′ R: 5′-AGCGTGTCCTTCCCCATCT-3′	585
SLA-2a	F: 5′-GCCATCCTCATTCTGCTGTC-3′ R: 5′-CGTGTCCTTCCCCATCTGC-3′	585
SLA-2b	F: 5′-GCCATCCTCATTCTGCTGTC-3′ R: 5′-AGCGTGTCCTTCCCCATCT-3′	587
SLA-3a	F: 5′-CCCGAGCCCTCTTCTTGCT-3′ R: 5′-TTTCTGGAGCCACACCACA-3′	566
SLA-3b	F: 5′-CCGAGCCCTCTTCCTGCTG-3′ R: 5′-TTTCTGGAGCCACTCCACA-3′	565
SLA-3c	F: 5′-GACCCTGGCCCTGACTGGT-3′ R: 5′-GGAGCCACTCCACACACGC-3′	533
SLA-3d	F: 5′-GACCCTGGCCCTGACTGGT-3′ R: 5′-GGAGCCACACCACACACGC-3′	533

PCR, polymerase chain reaction; F, forward; R, reverse.

[35] was used as a control for PCR correctness and isolate quality. The reaction mixture consisted of 1 U of *Taq* polymerase (Thermo Fisher Scientific), 25 mM $MgCl_2$, 0.53 mM each of dNTPs, 0.2 μM each of six primers, and 80 ng of genomic DNA isolated from porcine blood. Cycle conditions for multiplex PCR were 5 min of pre-incubation at 95°C, 40 cycles in three steps (94°C for 30 sec, 61°C for 30 sec, and 72°C for 40 sec), and finally, 72°C for 10 min. Products of reactions were separated by using 8% polyacrylamide gel electrophoresis with 3.5 M urea [41], followed by post-staining and analysis as described above.

Statistical analysis

The quantitative data were compared by using Student's *t*-test. For multiple comparisons, ANOVA was used. Analysis of unmeasurable variables was performed by using the χ^2 test. A p value < 0.05 was considered significant. All calculations were performed in Statistica software (ver. 12.0; StatSoft Polska, Poland).

Table 2. Characteristic of primers used for porcine endogenous retrovirus detection

Gene	Sequences	PCR product length (bp)
envA	F: 5′-GAGATGGAAAGATTGGCAACAGCG-3′	364
	R: 5′-AGTGATGTTAGGCTCAGTGGGGAC-3′	
envB	F: 5′-AATTCTCCTTTGTCAATTCCGGCCC-3′	270
	R: 5′-CCAGTACTTTATCGGGTCCCACTG-3′	
envC	F: 5′-CTGACCTGGATTAGAACTGGAAGC-3′	284
	R: 5′-GTTATGTTAGAGGATGGTCCTGGTC-3′	
Porcine *gapdh*	F: 5′-TGTCGCCATCAATGACCCC-3′	195*
	R: 5′-TGACAAGCTTCCCATTCTC-3′	116†

PCR, polymerase chain reaction; F, forward; R, reverse. *PCR product length when DNA is the template. †PCR product length when RNA is the template.

Results

PERV expression in transgenic and non-transgenic pigs

To study transcriptional activity of PERVs, the homologous sequence of the *gag* gene was analyzed. Due to > 90% identity at the nucleotide level among the subtypes of PERVs, the *gag* sequence allows holistic quantification of all types of PERV particles in porcine tissues. Our results showed that transgenic and non-transgenic pigs did not differ significantly in PERV *gag* RNA levels, likewise the type of applied transgene had largely no effect on PERV viremia (Table 3).

SLA class I typing

For the 50 herd specimens, *SLA* class I profiles were established (Fig. 2). The applied primers sets differed with the different specificity of the *SLA* class I complex sequences, allowing determination of 7 of the 8 identifiable *SLA-1* profile combinations (panel A in Fig. 3), all possible versions (four) of *SLA-2* genes (panel B in Fig. 3), and 15 of 16 *SLA-3* combinations (panel C in Fig. 3). The most often repeated *SLA-1* profile was *SLA-1ab*, found in 23.5% of the specimens. More than half of the specimens (54.9%) demonstrated a homologous *SLA-2* genes profile, *SLA-2ab*. The profile with the lowest frequency was *SLA-2b*, present in only 14% of specimens. The *SLA-3c* and *SLA-3ac* profiles were the most abundant *SLA-3* types within the herd (15.7% each).

PERV expression within *SLA* class I profile-based groups

Another question needing investigation was whether the *SLA* class I gene combinations may correspond with PERV level in blood and thereby influence infectiveness. The results, in turn,

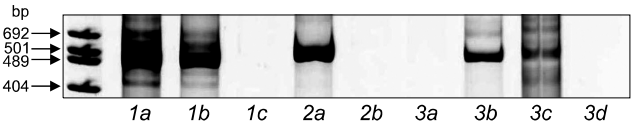

Fig. 2. A representative swine leukocyte antigen (*SLA*) class I pattern of an analyzed porcine blood sample. This specimen represents the *SLA* class I profile, *SLA-1ab 2a 3bc* (polymerase chain reaction products: 548, 548, 585, 565, and 533 bp, respectively).

Table 3. Descriptive statistics of porcine endogenous retrovirus viremia (viral particles per milliliter of blood) within the studied herd

Type of transgene	Median	Minimum	Maximum	Lower quartile	Upper quartile
None	1.45×10^8	1.28×10^7	9.60×10^8	8.81×10^7	3.59×10^8
GLA	5.95×10^7	8.48×10^5	3.35×10^9	3.43×10^7	1.88×10^8
FUT 2	2.07×10^8	3.09×10^4	1.58×10^9	7.95×10^6	4.42×10^8
GLA + FUT 2	1.95×10^7	4.47×10^6	1.28×10^8	5.26×10^6	8.05×10^6

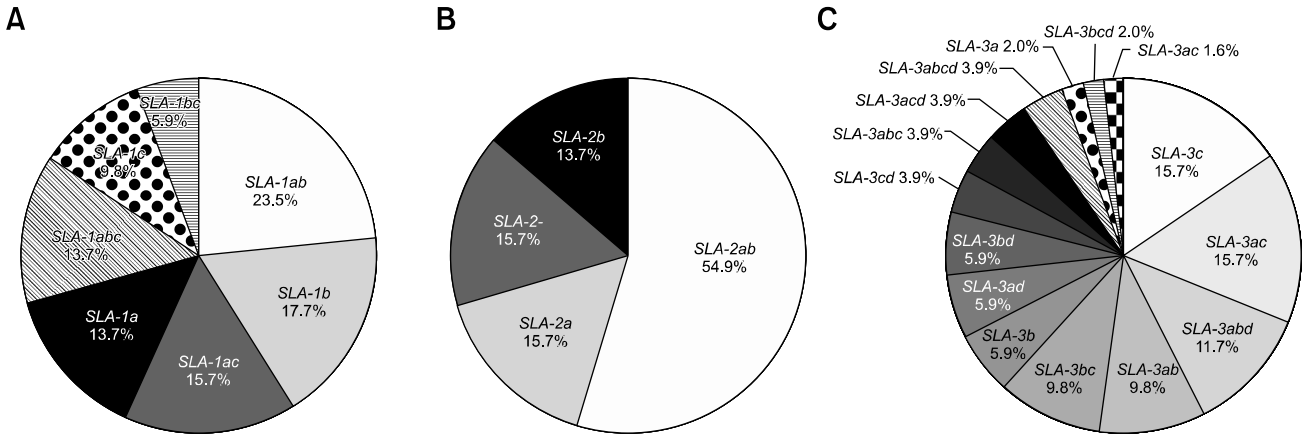

Fig. 3. Percentile share of swine leukocyte antigen (*SLA*) class I-based groups within the investigated herd. Clustering based on *SLA-1* (A), *SLA-2* (B), and *SLA-3* (C) profiles. Within the investigated herd there were 7 of 8 identifiable combinations of the *SLA-1* profile, four (all possible) versions of *SLA-2* genes, and 14 of 16 possible combinations of *SLA-3*. The most often repetitive *SLA-1* profile was *SLA-1ab*, found in 23.5% of the analyzed specimens. More than half of the herd (54.9%) demonstrated a homologous *SLA-2* genes profile: *SLA-2ab* and *SLA-3c* profiles, together with *SLA-3ac*, the most abundantly occurring profile among the herd (15.7% each).

could have adverse implications for xenograft recipient safety. Statistically significant differences in PERV *gag* RNA level were observed only between the *SLA-1* profile-based groups (panel A in Fig. 4). Among the *SLA-2* (panel B in Fig. 4) and *SLA-3* profiles (panel C in Fig. 4), no significant correlations to the quantity of PERV particles were detected ($p = 0.781$ and $p = 0.103$, respectively).

Screening of pig herd for PERV subtypes

In this study, multiplex PCR with *env*-specific primers was undertaken (Fig. 5). Less than one-third of the tested specimens (31.25%) showed the presence of PERV-C in the genome, and that presence was not associated with increased viremia. Distribution of particular *SLA* class I profiles, within groups and with/without the PERV-C subtype, was diverse (Fig. 6). Within the PERV-C subtype-positive group, the highest frequency occurred in *SLA* class I profiles: *SLA-1*, ab; *SLA-2*, ab; and *SLA-3*, c. The *SLA-1ab* and *SLA-3c* profiles occurred almost twice as often in the PERV-C-positive specimens (Fig. 6). Additionally, the *SLA-3bc* profile was observed to be correlated with the presence of the PERV-C subtype.

Discussion

The goal of this study was to determine the distribution of MHC class I alleles within a porcine herd and the prevalence of PERV viremia of the sampled specimens by using qualitative and quantitative PCR, respectively. Estimation of the interrelationships between transgene type, carried by several specimens and PERV load level and PERV subtype was also undertaken. The study showed no difference in PERV *gag* levels in blood samples of transgenic and non-transgenic pigs; as well, the type of introduced gene had no impact on viremia intensity. We placed special emphasis on investigating the relationship between *SLA* class I profile and PERV quantity in peripheral blood samples. The presence of several profiles (especially *SLA-1bc*) may result in an increased level of PERV particles.

Knowing the distribution of *SLA* class I alleles types occurring within a herd may have great importance in pathogen-free breeding programs [11,13,43]. Unfortunately, all PERVs cannot be eliminated due to their presence in germ-line DNA, but, as shown by the results, their level in peripheral blood can be associated with the animal's *SLA-1* profile. It has also been suggested that heterozygosity at the *SLA* class I region is related to enhancing resistance to infectious diseases by increasing the diversity of antigens presented to T cells and following the generation of a diverse T cell set [9,28], which can improve survival fitness remarkably and make animal tissues and organs safer for xenograft recipients. However, a report by Kwiatkowski *et al*. [16] showed an inhibitory influence of *SLA* proteins toward human NK cell-mediated cytotoxicity. Information about commonly occurring *SLA* alleles within a herd (or characterizing specimens living in a specific region) could be also important in providing immune protection in swine through vaccine development against viral pathogens such as swine influenza or porcine circovirus 2b [10,27]. Preliminary results have also shown the usefulness of *SLA* typing for genetic marker detection in an application related to pig disease-resistance breeding [10,43]. Moreover, some combinations of *SLA* class I and II haplotypes may be advantageous in providing immune responses against common and/or regionally endemic pathogens [12].

In the case of infection by some viruses (*e.g.*, human

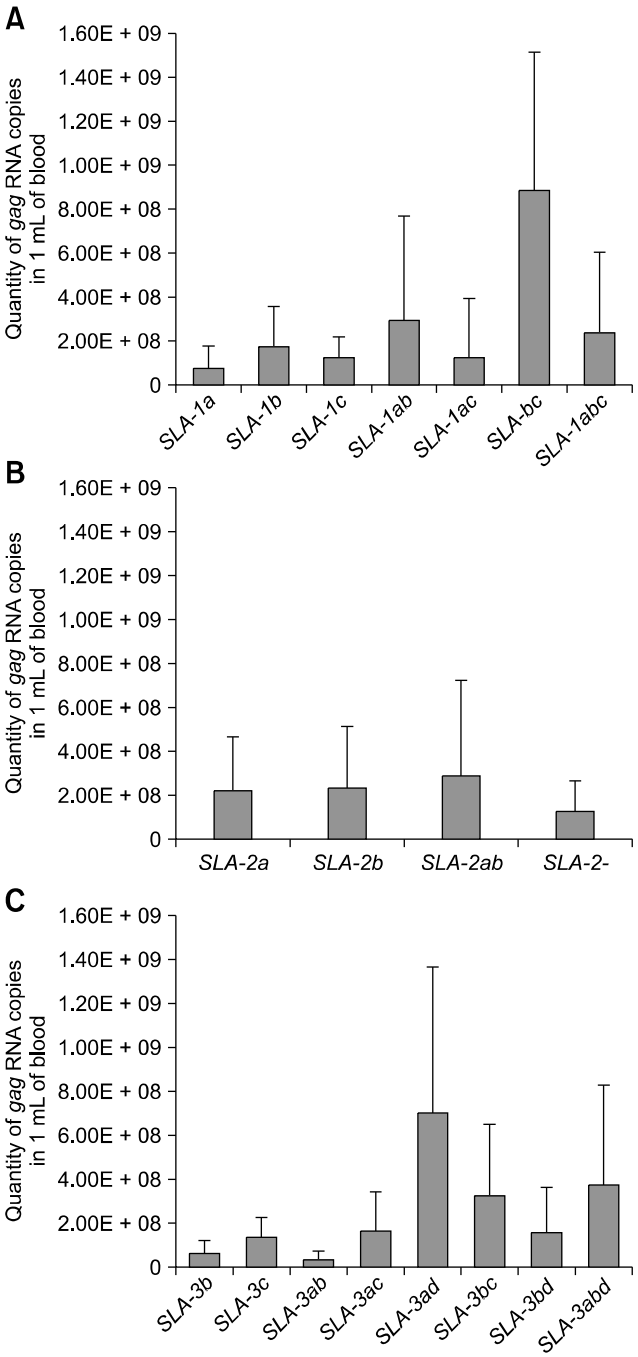

Fig. 4. Average porcine endogenous retrovirus (PERV) viremia within *SLA-1* (A), *SLA-2* (B), and *SLA-3* (C) profile-based groups. Statistically significant differences in PERV *gag* RNA level were observed only in the *SLA-1* profile-based group (A). *SLA-1bc* group displayed significantly higher viremia than that in the *SLA-1a*, *1b*, *1c*, and *1ac* profiles. No correlation with PERV viremia and the *SLA-2* (B) or *SLA-3* profiles (C) was detected. *SLA*, swine leukocyte antigen.

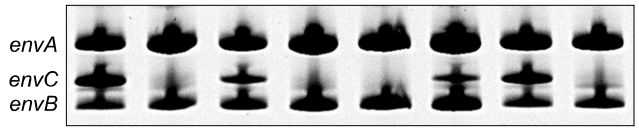

Fig. 5. Representative images of multiplex polymerase chain reaction (PCR) results for porcine endogenous retrovirus (PERV)-A, -B, and -C screening. Multiplex PCR with *env*-specific primers with denaturing polyacrylamide gel electrophoresis was conducted to detect the subtypes of PERV present in genome of pigs. As a result, 3 bands (364, 284, and 270 bp length) were obtained for PERV-C–positive specimens and 2 bands (364 and 270 bp length) for pigs without the PERV-C subtype in their genome. Less than one-third of the herd (31.25%) showed the presence of PERV-C in their genome, and no association with increased viremia was detected.

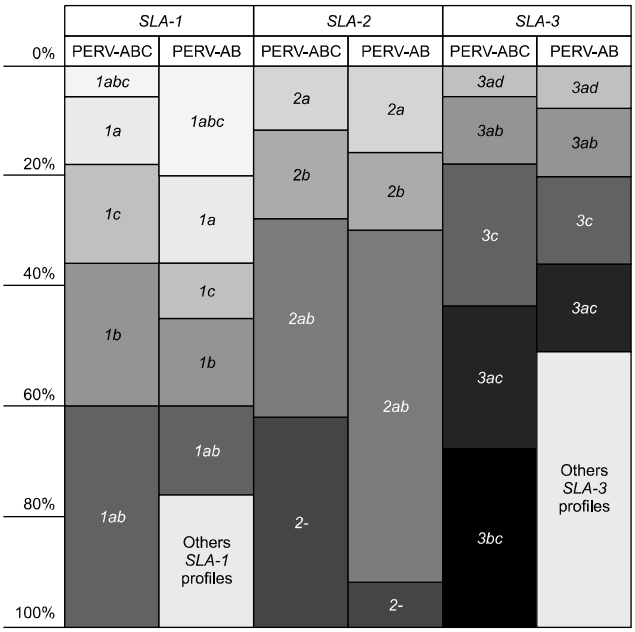

Fig. 6. Distribution of particular swine leukocyte antigen (*SLA*) class I profiles within the assessed herd. Classes were divided into two subgroups: with and without porcine endogenous retrovirus (PERV)-C subtype in their genome (left and right column, respectively, for each hue). In the PERV-C–positive group, the highest occurrences were in the following *SLA* class I profiles: *SLA-1*, ab; *SLA-2*, ab; and *SLA-3*, c. The *SLA-1ab* and *SLA-3c* profiles were present almost twice as often in the PERV-C –positive specimens than in the PERV-c–negative specimens.

immunodeficiency virus [HIV], hepatitis B virus, and hepatitis C virus), there are close relationships between the HLA-I profile of infected cells, the killer cell immunoglobulin-like receptors (KIRs) molecule profiles on NK cells, and the progression of infection [14,30,44]. In addition, it has been noted that the HIV can modulate the expression of selected HLA-I molecules on infected cells and, by that mechanism, influence viremia [22]. Analogously, interactions between receptors on human T cells and the ligand molecules on

SLA-expressed cells (*SLA* of transplanted cells, including those infected with PERV and KIRs on NK cells of the xenograft recipient), may determine transplant rejection as well as viremia levels of transduced PERV molecules.

SLA class I genes are highly polymorphic with 223 alleles being designated by 2013 [12], and that number is still increasing. The nature of *SLA* genes precludes rapid identification of their profile in a one-step test; as well, using serological methods is difficult due to insufficient availability of suitable sera that could be used to characterize all antigens. Moreover, *SLA* genes share similar outer epitopes with different MHC molecules [13,23]. Locus-specific sequencing of *SLA* genes would be the most precise technique, and could provide information about each nucleotide within a defined DNA fragment; however, the price and time-consumption of that approach would limit its application remarkably [5,23,29,40]. Other nucleic acid-based and allele-specific methods, such as sequence-specific primer PCR (SSP-PCR) and sequence-specific oligonucleotide probe PCR (SSOP-PCR), test for specific sites of known polymorphisms. However, those polymorphic sequence motifs may be common for other polymorphic *loci*, which makes those PCR primers nonspecific for a particular allele. Thus, although identification of one *SLA* allele by PCR-SSP may require more than one primer pair, that approach would require a lower number than the probe quantity required for *SLA* identification via PCR-SSOP [23]. When screening a herd, a crude-level analysis might turn out to be more appropriate because it could relatively quickly identify certain tendencies within the herd. In this study, we demonstrated that the *SLA-1* profile is correlated with the PERV particle level in blood samples and that pigs with the *SLA-1bc* and *SLA-1ab* profile have heightened viremia (Fig. 4). Thus, recipients of organs derived from such animals would be exposed to greater peril than those receiving organs from donors with other *SLA-1* profiles.

Another correlation was assessed during our analysis of *SLA* class I profiles and the distribution of PERV-C subtypes within the herd; however, it is unclear, how the presence of the PERV-C subtype is correlated with the MHC class I profile of pigs. Nevertheless, we observed a significantly increased proportion of PERV-C−positive individuals in the group with *SLA-1ab* and *SLA-3bc* profiles.

Screening tests for a herd intended for use in xenotransplantation application should allow the selection of animals with low PERV expression and should enable exclusion of specimens with PERV-C in the genome, thereby preventing recombination between the PERV-A and PERV-C subtypes [3,8]. The PERV-A/C forms are absent in the porcine germ-line and are produced by recombination; moreover, they can *de novo* integrate into the genome (autoinfection) [3,32,39]. Acquisition of the receptor binding site of PERV-A enables the recombinant C subtype virus to infect human cells, despite prior ecotropism. Changes in the structure of PERV-A/C genes, compared to their initial forms, are also associated with multimerization of binding sites (ATTGG) for transcription factor NF-Y in the long terminal repeat (LTR) sequences, which results in a significant increase in the PERV titer [6,7,15]. Such adaptation of the virus, including the LTR modification, was observed during passages of the PERV-A subtype, but it concerned repetition of different nucleotide sequences [7,31]. Frequency of passages, in turn, have been used to determine the direction of PERV evolution. Rapid passages preceding long-term culture in the absence of passage contribute to high-titer PERV-A/C recombinants with longer LTR sequences (due to high selection pressure), whereas mutation and deletion occur during long-term culture of cells [15]. Possible infection and evolution of PERVs in human cells after xenograft transplantation are a potential threat due to enhancement of retroviral pathogenicity, as was shown for HIV, murine leukemia virus, and feline leukemia virus [6,8]. Increased replication of PERVs in human cells may be associated with a higher probability of insertion mutagenesis (which could lead to oncogenesis) or enhanced progression of immunodeficiency. The transmission and co-replication of PERV-C with the -A or -B subtypes indicate probable recombination between PERVs and human retroviruses or related sequences which, as a consequence, may result in creating more harmful forms [6,7].

For these reasons, complete characterization of the molecular aspects (including *SLA* typing, viremia estimation, and PERV subtype screening) of specimens intended as donors for xenografts is crucial for ensuring the safety of clinical xenotransplantation. Understanding complexity, polymorphism, and function of the *SLA* system and its correlations with various molecular factors may contribute to the development of pig as animal model for xenotransplantation research [1,40], as well as for research into porcine and human diseases.

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Conflict of Interest

The authors declare no conflicts of interest.

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