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Analysis of Natural Recombination in Porcine Endogenous Retrovirus Envelope Genes

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Human tropic Porcine Endogenous Retroviruses (PERVs) are the major concern in zoonosis for xenotransplantation because PERVs cannot be eliminated by specific pathogen-free breeding. Recently, a PERV A/C recombinant with PERV-C bearing PERV-A gp70 showed a higher infectivity (approximately 500-fold) to human cells than PERV-A. Additionally, the chance of recombination between PERVs and HERVs is frequently stated as another risk of xenografting. Overcoming zoonotic barriers in xenotransplantation is more complicated by recombination. To achieve successful xenotransplantation, studies on the recombination in PERVs are important. Here, we cloned and sequenced proviral PERV *env* sequences from pig gDNAs to analyze natural recombination. The envelope is the most important element in retroviruses as a pivotal determinant of host tropisms. As a result, a total of 164 PERV envelope genes were cloned from pigs (four conventional pigs and two miniature pigs). Distribution analysis and recombination analysis of PERVs were performed. Among them, five A/B recombinant clones were identified. Based on our analysis, we determined the minimum natural recombination frequency among PERVs to be 3%. Although a functional recombinant envelope clone was not found, our data evidently show that the recombination event among PERVs may occur naturally in pigs with a rather high possibility.

Keywords: Porcine endogenous retrovirus, natural recombination, envelope, xenotransplantation, pig

The risk of zoonotic infection discourages the hope for substituting human organs with pig organs. Zoonosis in xenografts became an issue after the findings that PERVs could infect human cells *in vitro* [13]. However, PERV *in vivo* transmission has not been found yet in nonhuman primates [16] or human patients exposed to various porcine sources [12].

PERVs have been classified into the retroviral β and γ genera [21]. Infectious human tropic PERVs have been assigned to the PERV γ1 group consisting of the subgroups A, B, and C according to *env* sequences [8]. PERV-A and -B have wide host ranges *in vitro*. In contrast, PERV-C infects only pig cell lines [19].

All natural organisms undergo numerous genetic changes. In particular, the mutational rate of viruses is superior to other living organisms. Moreover, plentiful genetic alterations in the PERV genomes have happened and will be happening continuously. The major reason is that RNA-dependent DNA polymerase has a high error rate when reverse transcribing RNA into DNA, unlike other DNA-dependent DNA polymerases. It has no proofreading ability. Although most of the isolated PERVs in pig genome present as non-functional [6], the possibility of gain-function mutation will not be excluded. Additionally, the replication competence of PERVs is shown to be relatively higher than that of other endogenous retroviruses. Xenotransplantation might make PERVs cause new epidemics such as Avian Influenza viruses and Severe Acute Respiratory Syndrome viruses.

In recent times, an interesting finding about the risk of recombination was reported. A PERV A/C recombinant was approximately 500-fold more infectious to human cells than PERV-A [3]. Moreover, the possibility of recombination between PERVs and HERVs is often proclaimed as a risk of xenografting. To date, there has been no report about such recombination. No cross-packaging between PERVs and HERVs [17] gives a small chance of recombination. Nevertheless, the risk of recombination cannot not be ignored.

Under these circumstances, recombination study is necessary for safe xenotransplantation. Additionally, the potential for recombination of PERVs with other viruses or other PERVs is also necessary to monitor and examine. To

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evaluate the risk of recombination in PERVs, we analyzed the proviral PERV *env* sequences, the most important component as a determinant of host infectivity, from pig genomic DNAs (from four conventional pigs and two miniature pigs). Molecular characterization of recombinants and estimation of the natural recombination frequency among PERVs in pigs were performed.

Materials and Methods

Isolation of Pig Genomic DNA

Genomic DNA was isolated from pig hair roots and peripheral blood mononuclear cells (PBMCs) using the QIAamp Mini Kit (Qiagen). Hair roots were collected from four conventional pig breeds (five pigs each of Berkshire, Duroc, Landrace, and Yorkshire breeds, 20 total) and PBMCs were obtained from two types of miniature pigs (Middle, M; and Tiny, T) in Korea. Blood samples of miniature pigs were kindly provided by PWG Genetics Korea in Pyung-taek. The M strain is a medium-size miniature pig having a birth body weight of 0.47 kg. The T strain is a tiny-size miniature pig having a birth body weight of 0.21 kg. At the time of bleeding, the body weight of M and T was 18 and 11 kg, respectively.

PCR and Cloning of the PERV *env* Gene

The primer set used for PCR includes the conserved region of the PERV *env* gene, based on the submitted nucleotide sequences of PERV *env* genes in the GenBank database. Total of 82 sequences (representative sequences: PERV-A, AY312524, and AY312526; PERV-B, AY312522, and AY312531; PERV-C, AF038600, and AF402661) were analyzed for designing primers, which were used to clone all subgroups of PERVs. PCR was performed with forward primer 5'-ACCTGGATCCATGCATCCCACGTTA A-3' and reverse primer 5'-(A/G)TCTGA AGTGGTTCTACA- GAAC(A/C)G(A/G)A-3', including the start codon through part of p15E of the PERV *env* sequence. PCR condition was one cycle of 94°C for 3 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and one cycle of 72°C for 7 min. Approximately 1.6-kb PCR products were purified using a gel extraction kit (Qiagen) and then cloned into a pCR2.1-TOPO vector (Invitrogen).

Analysis of *env* Sequences

All clones were sequenced using an ABI sequencer. To estimate the level of divergence between PERVs, their nucleotide sequences were aligned. Phylogenetic trees were generated using the neighbor-joining method by a computer program TREECON 1.3b and MegAlign in the Lasegene sequences analysis software package [4, 5]. The reliability of the neighbor-joining topologies was estimated by performing 100 bootstrap replicates. The following sequences were used as references, with GenBank accession numbers in parentheses: PERV-A (AY312524, AY312526, AF130444, AY312527, and AF507940), PERV-B (AY312522, AY312531, AJ133818, AJ288587, and AJ288590), PERV-C (AF038600 and AF402661), and PERV-E (AF356698).

To certify the recombinants, the methodology given at the Web site http://www.ncbi.nih.gov/projects/genotyping/ was also used for classification of subgroups and screening of recombinant sequences [14]. This method is more helpful than the phylogenetic analysis, which could not distinguish the mosaic organization of recombinant viruses by multiple alignments of a query sequence. The following strains were used as references. PERV-A: AF435967, AY570980, AF426923, AF426944, AF426942, AF426934, AF426941, AF426929, AF426917, and AF426931; PERV-B: AY099324, AF426938, AF426946, AF426937, AF426916, AY312530, AJ288590, AJ288589, AJ288591, and AJ288587; PERV-C: AF038600, AY534304, and AY534305; and PERV-E: AF356698.

Results and Discussion

Distribution of Proviral PERVs

To evaluate the risk of recombination in PERVs, we cloned the proviral envelope genes from four conventional pigs and two miniature pigs by the PCR method. A total of 164 envelope clones were isolated and sequenced. As similarly reported in another study, most of the isolates were PERV-A and B with similar distributions (47.5% of PERV-A and 42.6% of PERV-B) compared with a minor portion of PERV-C (6.7%) [9]. Out of 164 isolates, we found five unidentified clones under the procedure of simple alignment (Table 1). The two clones were isolated from Berkshire and Duroc each, and one clone from a miniature pig. Intrabreed distribution of each breed showed differences caused by sampling size. Despite differences among breeds, approximately 90% of total clones were human tropic PERV-A and PERV-B.

Table 1. PERV *env* clones analyzed in this study.

	Strain	Subgroup				Total
		PERV-A	PERV-B	PERV-C	Unidentified (Recombinant)	
Conventional pigs	Berkshire	10	6	-	2	18
	Duroc	11	9	1	2	23
	Landrace	14	10	-	-	24
	Yorkshire	19	13	-	-	32
Miniature pigs	M	10	16	4	-	30
	T	14	16	6	1	37
Total		78	70	11	5	164

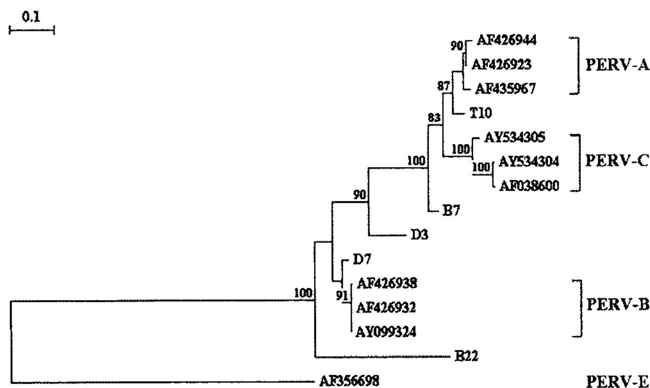

Fig. 1. Neighbor-joining tree based on the nucleotide sequences of the recombinant PERV *env* gene from Korea.

The tree was generated using the method of Kimura, by a computer program TREECON 1.3b. Numbers at the nodes indicate the bootstrap value of 100 resampled datasets. The following sequences were used as references. PERV-A: AF426944, AF426923, and AF435967; PERV-B: AF426938, AF426932, and AY099324; PERV-C: AY534305, AY534304, and AF038600; PERV-E: AF356698.

Recombinant Screening

Molecular phylogenetic analyses were performed with five unidentified clones and 10 reference strains. Approximately 1.6-kb envelope sequences were aligned using CLUSTRAL X 1.8 [20] with a series of reference strains (A to C and E) from the GenBank sequence database. A phylogenetic tree was generated (Fig. 1). T10, isolated from the miniature pig, was located between PERV-A and -C with 83% bootstrap value. D7 from Duroc was located closely to PERV-B, with lower than 80% bootstrap value. B7 and D3, from Berkshire and Duroc, respectively, were clustered closely related to PERV-A and -C. B22 from Berkshire was out-grouped with PERV-A, -B, and -C with 100% bootstrap value. Using phylogenetic analysis, we identified five recombinant candidates within our isolates.

The Web-based genotyping tool supplied by NCBI was used for screening the recombinant sequences. We analyzed all cloned sequences using this tool to search recombinants having mosaic organization (data not shown). Although five unidentified clones showed profiles corresponding to recombinants, there was no mosaic recombinant in our clones. By dual analysis, we confirmed that five clones were definitely the recombinants.

Molecular Analysis of Recombinant Clones

Recombinants were classified by recombination site and recombination pattern. According to the recombination site, four of five recombinant clones were recombined in the region of gp70, and D7 clone alone was recombined after the cleavage site of gp70 and p15E. By recombination pattern, four of five clones represented homologous recombination with single crossing over between PERV-A and -B. B22 clone alone showed an inverted pattern of PERV-B and -A. All clones were B/A recombinants (Fig. 2).

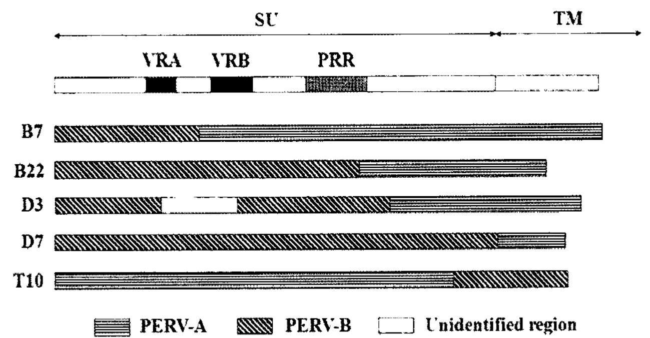

Fig. 2. Schematic description of recombination patterns in PERV *env*.

The boxes represent PERV-A (horizontal line), -B (diagonal line), and unidentified region (open box). Functional motifs of *env* are indicated at the top: SU, surface glycoprotein; TM, transmembrane region; VRA, variable region A; VRB, variable region B; PRR, proline-rich region. Origin of sequences: B7 and B22 from Berkshire; D3 and D7 from Duroc; T10 from miniature pig T strain.

In B7, a single crossing over occurred at sequence CCAGGACC between PERV-B (450th bp to 457th bp) and PERV-A (349th bp to 356th bp). The location of nucleotide sequence is indicated by the reference sequence in Fig. 3. In D3, one crossing over was detected at sequence TATGAGGG between PERV-B (1,000th bp to 1,007th bp) and -A (1,009th bp to 1,016th bp); in D7, TATCCCT between PERV-B (1,384th bp to 1,390th bp) and -A (1,393rd bp to 1,399th bp); in T10, TTAACCCC between PERV-A (1,245th bp to 1,252nd bp) and -B (1,236th bp to 1,243rd bp).

In B22, the fragment from ATG to the 910th bp was PERV-B and that from the 901st bp to the 1,488th bp

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                      428                     450     457                    479
AF426938  PERV-B      ACTGGAAATGGCCGATCTCTCT  CCAGGACC  GGGTAAAATTCTCCTTTGTCAA
B7        PERV-B/A    ACTGGAAATGGCCGATCTCTCT  CCAGGACC  CCCAAATAATGAAGAATATTGT
AF435967  PERV-A      TGCTTACGGGTTTTACGTTTGC  CCAGGACC  CCCAAATAATGAAGAATATTGT
                      327                     349     356                    378

                      876                      901 902                       927
AY099324  PERV-B      TGTTCCTGTTAAGACAGGACAGAGAC | TCTTCAGTCTCATCCAGGGAGCTTTC
B22       PERV-B/A    TGTTCCTGTTAAGACAGGACAGAGAC | CAGCTTATTTGCTTATTTTGTACATC
AF426944  PERV-A      TTAAGTAATCTAGGTCTAACAAATTA | CAGCTTATTTGCTTATTTCGTACATC
                      613                      588 587                       562

                      978                    1000     1007                  1029
AF426938  PERV-B      TCTATCCTCAGGGCCTCCTTAT  TATGAGGG  GATGGCTAAAGAAGGAAAATTC
D3        PERV-B/A    TCTATCCTCAGGGCCTCCTTAT  TATGAGGG  AATGGCTAGAGAAGGAAAATTC
AF426923  PERV-A      CTTAGCTTTGGGCCCACCTTAC  TATGAGGG  AATGGCTAGAGGAGGGAAATTC
                      987                    1009     1016                  1038

                      1362                   1384   1390                    1413
AF426938  PERV-B      AACCGACCAAAAAGAGAACCCG  TATCCCT  TACCCTAGCTGTAATGCTCGGAT
D7        PERV-B/A    AACCGACCAAAAAGAGAACCCG  TATCCCT  GACACTAGCTGTAATGCTCGGAC
AF426923  PERV-A      AATCGGCCAAAAAGAGAACCCA  TATCCCT  GACACTAGCTGTAATGCTCGGAT
                      1372                   1393   1399                    1423

                      1214                   1236   1243                    1265
AF426938  PERV-B      AACCGACCAAAAAGAGAACCCG  TATCCCT  TACCCTAGCTGTAATGCTCGGAT
T10       PERV-A/B    AACCGACCAAAAAGAGAACCCG  TATCCCT  GACACTAGCTGTAATGCTCGGAC
AF426923  PERV-A      AATCGGCCAAAAAGAGAACCCA  TATCCCT  GACACTAGCTGTAATGCTCGGAT
                      1223                   1245   1252                    1274
```

20

Fig. 3. Sequence comparison of the clones B7, B22, D3, D7, and T10 to reference strains of PERV: AF426938, AF426944, AY099324 (PERV-B), AF435967, AF426923 (PERV-A).

Recombinant sequences are described in the middle. Recombination joint sequences are shown in the box with dotted line. Lines are drawn to divide off homologous regions with references. Arrows indicate the direction from the ATG start codon. The sequence position is indicated below and above the reference sequences.

was PERV-A (which corresponded to an inverted fragment from PERV-A ATG to 587th bp) (Figs. 2 and 3). Inversion by recombination around the origin was postulated and experimented in prokaryotes [7]. In eukaryotes, pericentric recombination having a centromere between two recombination sites resulted in four products: one normal, one inversion product, and two duplication/deletion products [18]. B22 was thought to be one of the inversion or duplication/deletion products. Owing to the lack of information on the chromosomal structure and orientation of PERV-A and -B as proviruses before recombination, and because the *env* sequence by itself did not give sufficient evidence, it was impossible to establish which of the three was the case for B22. Nonetheless, both ends of B22 had the start codon, and we could not find recombination joint sequences for homologous recombination. At the crossing over site, we only found a symmetric sequence, GACCAG.

Recombination and Envelope Function

B7 clone showed asymmetric crossing over between PERV-B and -A. Asymmetric crossing over would contribute to a change in molecular mass by adding 33 amino acids at the receptor binding domain (RBD) (Fig. 4B). Considering the critical roles of gp70, recombination in B7 could confer new characteristics in tropism and infectivity.

Oldmixon *et al.* [11] reported an interesting recombinant. This recombination enabled the A/C recombinant to infect human cells and to be more infectious than PERV-A. If B7 clones had the correct ORF, it would have some different characteristics in infectivity and host tropism compared with PERV-A or -B. Because of premature termination, sequence modification was required to study the relationship between recombination and cell infectivity.

Recombination Relation to Glycosylation

The PERV envelope is a glycoprotein. For example, PERV-A (Y12238) has 9 potential *N*-glycosylation sites, -B (Y12239) has six, and -C (AF038600) has eight. Glycosylation is very important in binding to the host receptor [1, 15]. Fig. 4 presents the schematic description of potential glycosylation sites in recombinants. Two clones (B22 and D7) were excluded in this figure because B22 did not make complete *env* by inverted pattern, and D7 recombined at the outside of gp70. As a result of recombination, there was no difference in glycosylation in B7 and T10 compared with PERV-A. In D3, the position and number of glycosylation were changed. RBD of

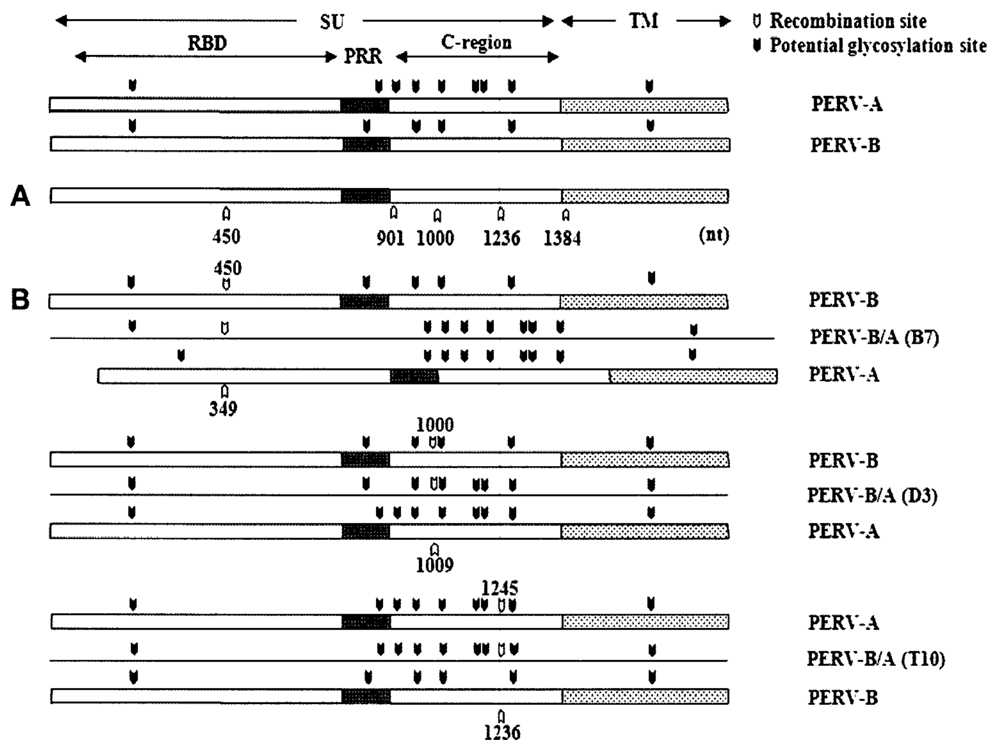

Fig. 4. Schematic description of recombination in PERV *env* related to potential glycosylation sites.
The functional motifs of *env* are indicated at the top. SU, surface glycoprotein; TM, transmembrane region; RBD, receptor binding domain; PRR, proline-rich region; C-region, constant-region. Dark arrows indicate potential glycosylation site. Open arrows indicate recombination site. The sequence position is indicated below and above the arrows. Y12238 (PERV-A) and Y12239 (PERV-B) were used as reference sequences. **A.** Although a recombination hot-spot was not found in the PERV envelope gene by analyzing all recombinant *env* sequences submitted in GenBank (data not shown), the C-region was more frequently recombined than RBD based on our data. **B.** Recombination patterns related to glycosylation were analyzed. B7 showed interesting asymmetric recombination in the receptor binding domain. As a result of recombination, D3 showed changes in the position and number of glycosylation compared with PERV-A.

D3 was PERV-B and the rear of PRR was PERV-A. Thus, it was expected that conformational changes by recombination concerning glycosylation (for example, by the effect of steric hindrance) might show the change of virus characteristics.

Until now, neither the genuine glycosylation site nor the glycosylation site significant to a binding property has been reported. Therefore, researches related to glycosylation and recombination are indispensable for understanding the PERV infection mechanism.

Recently, PERV-A receptors were identified in humans and some other vertebrates [2], and transgenic mice study using these receptors was performed [10]. However, the receptors of PERV-B and -C have not been identified yet. In other viruses, glycosylation in host receptor was important for its interaction with virus envelope protein [1, 15]. In an evolutionary perspective, the host modifies glycosylation of receptor proteins for antiviral immune reaction; on the other hand, the virus modifies glycosylation of the envelope for survival. Regarding PERVs, information about the mechanism of their infection (receptors, glycosylation studies of receptor and viral envelope, interaction of receptor and viral envelope, and so on) to human cells has not been sufficient.

Natural Recombination Rate

In our study, five of 164 clones were identified as recombinants: the valuated natural recombination frequency in our study was at least 3%. In fact, there would be more recombinant clones in natural porcine genome.

There were several reasons for setting the natural recombination frequency higher than 3%. Firstly, the natural recombination rate among PERVs could increase considerably because of recombinants in other regions (LTR, *gag*, and *pol*) having high homology. Secondly, we had no methodological tool to find the recombination between inter-subgroups (for example, crossing over between PERV-A and -A, between PERV-B and -B, and so on.). Specifically, we could not perfectly distinguish the recombination between PERV-A and -C, if their crossing over point was not located in the variable region. Except for the variable region, PERV-A and -C have great homology with each other. Lastly, recombinants, not amplified by our primer sets in spite of primers designed for all PERVs, might exist. Moreover, in the *env* region, other recombinants could occur in the remaining portion of p15E, excluded in cloning. Owing to the importance and high variation of glycoproteins, we focused on the cloning and analysis of gp70 to evaluate the minimum natural recombination frequency.

Recombination with PERV-C

Because of the low copy numbers and recombination rates, we could not find any PERV-C recombinant in our isolates. Within our isolates, the distribution of PERV A: B: C was 47.6: 42.7: 6.7, and the percentage of recombination between PERV-A and -B was 3%. Based on these circumstances, the possibility of recombination between PERV-C and other PERVs (-A or -B) was estimated to be less than 0.3%.

D3 had an unidentified region of about 300 bp, as noted in Fig. 2 as the open box. When a Blast search was done with sequences of this region, a fragment of approximately 100 bp was 77% homologous with the PERV-C. However, we could not conclude that D3 was the result of recombination between PERV-B and -C. The length was too short and the homology between PERV-A and -C was too high. Additionally, insufficient pig genome information made it difficult to analyze this recombinant. Therefore, we decided to keep this region as unidentified.

The report of Wood *et al.* [22] gave another point of view in relation to PERV-C recombination. They showed that high-titer A/C recombinants [11] were exogenous viruses in miniature swine. However, our study was based on the provirus stably transmitted through germ lines. A low possibility of PERV-A/C recombination indicates that transmission of a high-titer PERV-A/C recombinant through germ lines is a rare event. To obtain a recombinant with PERV-C, a large sampling size would be required.

Our study showed that recombination among PERVs could occur in pig naturally above 3%. High nucleotide sequence homology in PERVs could enable recombination with a rather high possibility. According to our study on the recombination in gp70 of PERV *env*, more recombination events occurred in the C-region than the variable region and PRR because of the relative high homology in gp70 (Fig. 4A). To find out the recombination preference in the PERV envelope region, we analyzed all *env* sequences in GenBank. However, a recombination hot-spot was not found, with the same reason (data not shown). Despite the high possibility of recombination, we could not find any functional recombinant and distinctive hot-spot. Nevertheless, recombination is another important risk in zoonosis. Additionally, we could not completely eliminate the risk of recombination with other viruses during or after xenotransplantation, for example, with HERVs. Recombination is a difficult and complex barrier for safe xenotransplantation. Further studies about recombination are required for the perfect control or elimination of PERVs.

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