CHARACTERISING ENDOGENOUS PORCINE RETROVIRUSES (PERVs)

J. H. Lee[1], J. S. Burgess[2], Y. Chen[1], C. Moran[1]

[1]Department of Animal Science, University of Sydney, NSW 2006
[2]National Pancreas Transplant Unit, Westmead Hospital, NSW 2146

SUMMARY

The safety of xenotransplantation using pig organs and tissues could be compromised by Porcine Endogenous Retroviruses (PERVs). PCR products from PERV *env* genes were cloned and sequenced, and 9 different types were found in the Westrans inbred line of pig. Five have stop codons in the *env* gene compared with published PERV-A and PERV-B sequences, and one type, designated c1, shows evidence of recombination at the border of the surface and transmembrane regions of the *env* gene.

Keywords: PERV, cloning, sequencing, *env* gene

INTRODUCTION

Pigs are being promoted as the best source of organs and tissues for transplantation into humans for safety, financial, ethical, and practical reasons. Until recently, the main apparent obstacle to xenotransplantation was the aggressive immune reaction, called hyperacute rejection (HAR). Various strategies are being developed to control HAR (Cozzi and White 1995). Recently, a new problem has emerged. Retroviruses, expressed from a pig kidney cell line (PK 15), were shown to infect different types of human cells (Patience *et al.* 1997). This suggested that the use of pig organs and tissues for xenotransplantation could be dangerous because of the endogenous retroviruses (PERVs). However, the limited available data suggest that there is no PERV infection *in vivo* (Heneine *et al.* 1998, Patience *et al.* 1998). Two types of pig retrovirus, PERV-A and PERV-B, with characteristic sequences for the envelope (*env*) gene, the major determinant of retrovirus tropism, have been shown to be widely distributed in different pig breeds and inherited as provirus (Le Tissier *et al.* 1997). We are characterising all PERVs in a line of pigs (Westrans line) that is being inbred at Westmead Hospital for transplantation research.

MATERIALS AND METHODS

Animal resources. The most inbred pig available from the Westrans (Westmead transplantation) line was chosen for analysis. The Westrans animals are partly derived from a small population of feral pigs from Kangaroo Island, which were deliberately released over a century and a half ago. Some of these feral pigs were captured and transferred to the Adelaide University for a breeding program for medical research but were crossed with commercial Large White pigs. After being maintained as a very small colony for about 10 years, a pair of full sibs was transferred to Westmead Hospital in Sydney for transplantation research. The core breeding line has been maintained by deliberate full-sib mating ever since. The most inbred animals are now in the 6th generation of recorded deliberate inbreeding. Their highly inbred status has been confirmed by microsatellite data (not shown).

Primer design. The published PERV-A and -B sequences (Le Tissier *et al.* 1997) were aligned using the Gap program (Australian National Genomic Information Service, http://www.angis.su.oz.au). The *env* genes of PERV-A and PERV-B have highly conserved regions at each end, flanking a

variable region which forms the basis of recognising the A and B variants. The primers were chosen from the highly conserved regions in order to search for novel variants in the less conserved region. The forward primer was 5'-CCAAGCATCCCACGTTAAGC-3', and the reverse primer was 5'-ACCATCCTTCAAACCACCC-3'.

PCR conditions. PCR was carried out in a 50µl volume with approximately 100ng of template genomic DNA, 0.2mM of each primer, 600µM of each dNTP, 2mM $MgCl_2$, 10×PCR buffer (Invitrogen), and 2.5 units of Taq polymerase. This mixture was overlaid with one drop of mineral oil. Amplification was performed in a MJ thermocycler for 45 cycles with denaturation for 1 min. at 94°C, annealing for 1 min. at 65°C and extension for 1 min. 72°C. The final extension was for 20 mins. at 72°C.

Screening and sequencing of PERV clones. The *env* PCR products were cloned using a TOPO-TA cloning kit according to the manufacturer's instructions (Invitrogen). Restriction enzymes (*Kpn*I and *Mbo*I) were used for preliminary screening of the clones for characteristic features of PERV-A and –B (Le Tissier *et al.* 1997). A *SequiTherm* EXCEL™ Long-Read™ DNA sequencing kit (Epicentre) and Li-Cor sequencer (Model 4200, Li-Cor Inc.) were used to read approximately 1.8kb of full length insert sequence, using vector primers (M13 Forward and M13 Reverse primers). Sequences were analysed using Base ImageIR™ software (Li-Cor Inc.).

RESULTS AND DISCUSSION

Sixty four positive PERV clones were picked, plasmids prepared and inserts digested by *Kpn*I or *Mbo*I. Four different restriction patterns were identified by *Kpn*I digestion and six types by *Mbo*I digestion (Fig 1). Altogether nine different types of clones were identified by restriction digestion.

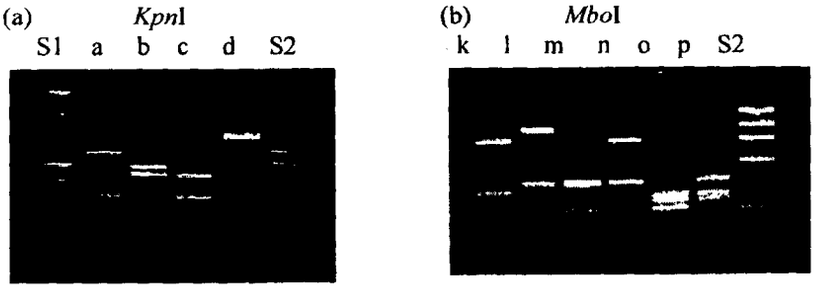

Figure 1. Restriction digestion of PERV clones. (a) Four patterns (a, b, c, d) of *Kpn*I digestion were identified and (b) six patterns (k, l, m, n, o, p) of *Mbo*I digestion. S1 and S2 are the 1kb DNA ladder (Promega) and ϕ×174DNA/*Hae* III Marker (Promega) size standards, respectively.

Table 1 shows nine different types of PERV clones identified in the inbred pig, based on digestion with the two restriction enzymes. Two clone types, am (16%) and bl (64%), are most common and their sequences were shown to correspond quite well to PERV-A and PERV-B respectively.

Comparison of the sequence of all 9 clone types showed that 5 corresponded to PERV-A and 4 corresponded to PERV-B, but that in some cases there were substantial mismatches with the cognate

sequence, indicative of a high level of heterogeneity in the PERVs. Although there have been some unpublished reports of a third type of PERV, no new type could be recognised which was as distinctive as PERV-A from PERV-B.

A sequence similarity matrix (Table 2) shows over 94% similarity within PERV-A and within PERV-B clones. However, there is only 76% to 80% sequence similarity between PERV-A and PERV-B types. This result suggests that the type A and type B classification is quite robust, despite an apparently high mutation rate within the *env* region.

Table 1. Restriction digestion and sequence analyses of PERV clones

Digestion pattern	No.	Most likely type from sequence comparison	Mismatches with most likely type
ak	1	A	100/1785
am	10	A	53/1785
an	4	A	52/1785
ao	1	A	53/1785
ap	4	A	52/1785
bl	41	B	14/1776
bn	1	B	8/1776
cl	1	B	100/1776
dl	1	B	9/1776

Table 2. Sequence similarity matrix of 9 PERV clones and 2 published sequences

Clone	sequence similarities										
	ak	am	an	ao	ap	A	bl	bn	cl	dl	B
ak	-										
am	94.7	-									
an	96.2	98.1	-								
ao	94.5	97.8	97.9	-							
ap	94.3	97.1	97.2	98.5	-						
A	94.1	97	97.1	97	97.1	-					
bl	79.6	76.7	76.6	76.5	76.8	76.8	-				
bn	79.7	76.7	76.6	76.5	76.7	76.9	98.7	-			
cl	79.6	79.5	79.5	79.2	79.6	80.1	93.8	94	-		
dl	79.9	76.7	76.7	76.6	76.7	76.9	98.6	99	93.9	-	
B	80	76.9	76.9	76.8	77	77.2	99.2	99.6	94.4	99.5	-

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          351                                                        400
cl-type   PPYYEGMAKE GKFNVTKEHR NQCTWGSRNK LTLTEVSGKG TCIGKVPPSH
PERV-A    ........RG .......... D......Q.. .......... ....M.....
PERV-B    .......... .......... .......... .......... .....A....

          401                                                        450
cl-type   QHLCNHTEAF NQTSESQYLV PGYDRWWACN TGLTPCVSTL VFNQTKDFCI
PERV-A    .......... .R........ .......... .......... .........V
PERV-B    ....YS.VVY E.A..N.... ...N..... .........S ....S....V

          451                                                        500
cl-type   MVQIVPRVYY YPEKAILDEY DYRNHRQKRE PISLTLAVML GLGVAAGVGT
PERV-A    .......... .......... ...YN.P... .......... ..........
PERV-B    .......... H..EVV.... ...YN.P... .V........ ...T.V....

          501                                                        550
cl-type   GTAALVTGPQ QLETGLSNLH RIVTEDLQAL EESVSNLEES LTSLSEVVLQ
PERV-A    .....I.... ...K...... .......... .K........ ..........
PERV-B    .....I.... ...K..GE.. ..M....R.. .......... ..........
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Figure 2. Part of the alignment of the deduced envelope protein sequence of PERV-A, PERV-B and the cl type. Up to amino acid 395, cl is identical to the PERV-B sequence but then swaps over to very close similarity with PERV-A. The predicted cleavage site between surface and transmembrane regions is marked with an arrow, on the basis of comparisons with murine leukaemia virus.

The mismatch pattern of the cl type, particularly in the deduced amino acid sequence (Fig 2) suggests that there may have been a recombination event between the A and B types near the border of the surface and transmembrane regions, as deduced from murine leukaemia virus (Battini *et al.* 1995), and that this has generated a new variant, which cannot be classified easily as type A or type B.

Five (ak, bl, an, ao, bn) out of 9 types of PERV clones identified in this study have stop codons within the envelope protein encoding region. These endogenous retroviruses could not make full length envelope protein and thus could not be recognised by cell surface receptor for the virus. Such PERVs are unlikely to constitute a hazard in xenotransplantation. It is perhaps reassuring to note that the most common clone type found in this study, namely the bl type, is for this reason unlikely to be functional. This conclusion must remain tentative until further bl clones are sequenced.

REFERENCES

Battini, J-L., Danos, O., Heard, J. M. (1995) *J. of Virology.* **69**(2):713-719

Cozzi, E. and White, D. J. G. (1995) *Nature Medicine* **1**(9):964-966

Heneine, W., Tibell, A., Switzer, W. M., Sandstorm, P., Vazquez Rosales, G., Mathews, A., Korsgren, O., Chapman, L. E., Folks, T. M., Groth, C. G. (1998) *The Lancet* **352**:695-698.

Patience, C., Takeuchi, Y. and Weiss R. A. (1997) *Nature Medicine* **3**(3):282-286

Patience, C., Patton G. S., Takeuchi, Y., Weiss, R. A., McClure, M. O., Rydberg, L., Breimer, M. E. (1998) *The Lancet* **352**:699-701

Le Tissier, P., Stoye, J. P., Takeuchi, Y., Patience, C. and Weiss R. (1997) *Nature* **389**:681-682