



## Analysis of Swine Leukocyte Antigen Haplotypes in Yucatan Miniature Pigs Used as Biomedical Model Animal

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**ABSTRACT:** The porcine major histocompatibility complex (MHC) is called swine leukocyte antigen (SLA), which controls immune responses and transplantation reactions. The SLA is mapped on pig chromosome 7 (SSC7) near the centromere. In this study, 3 class I (*SLA-1*, *SLA-3*, and *SLA-2*) and 3 class II (*DRB1*, *DQB1*, and *DQA*) genes were used for investigation of SLA haplotypes in Yucatan miniature pigs in Korea. This pig breed is a well-known model organism for biomedical research worldwide. The current study indicated that Korean Yucatan pig population had 3 Class I haplotypes (Lr-4.0, Lr-6.0, and Lr-25.0) and 3 class II haplotypes (Lr-0.5, Lr-0.7, and Lr-0.25). The combinations of SLA class I and II haplotype together, 2 homozygous (Lr-4.5/4.5 and Lr-6.7/6.7) and 3 heterozygous (Lr-4.5/6.7, Lr-4.5/25.25, and Lr-6.7/25.25) haplotypes were identified, including previously unidentified new heterozygous haplotypes (Lr-4.5/4.7). In addition, a new SLA allele typing method using Agilent 2100 bioanalyzer was developed that permitted more rapid identification of SLA haplotypes. These results will facilitate the breeding of SLA homozygous Yucatan pigs and will expedite the possible use of these pigs for the biomedical research, especially xenotransplantation research. (**Key Words:** Haplotype, Major Histocompatibility Complex [MHC], Swine Leukocyte Antigen [SLA], Yucatan Miniature Pig)

### INTRODUCTION

The major histocompatibility complex (MHC) region, containing a number of immune related genes, is one of the most significant genomic locations in animals due to its association with immune responses. The porcine MHC antigens, which is also called swine leukocyte antigen (SLA), recognize self and non-self peptides on the surface of T-lymphocytes and ultimately triggers the immune rejection cascade. Based on their biological functions, the MHC antigens are divided into 3 classes, SLA class I, II, and III, respectively. Most nucleated cells can express SLA class I

genes that are recognized by CD8+ T cells (Shishido et al., 1997). SLA class II genes are expressed on antigen presenting cells and are recognized by CD4+ T cells. On the other hand, SLA class III genes have several different functions, some of which are related to the immune response called complement cascade (Naziruddin et al., 1998). In 2002, SLA Nomenclature Committee of the International Society for Animal Genetics (ISAG) established the systematic nomenclature of the class I and class II SLA alleles (Smith et al., 2005a, b; Ho et al., 2009a, b; Ho et al., 2010). The recent updates of SLA alleles can also be found in the Immuno Polymorphism Database-Major Histocompatibility Complex (IPD-MHC) website ([http:// www.ebi.ac.uk/ipd/mhc/sla/](http://www.ebi.ac.uk/ipd/mhc/sla/)).

The SLA genes are highly polymorphic and play very important roles in regulating the immune system against infectious diseases. The relationships between SLA haplotypes and immune responses to various infectious diseases are previously reported (Lunney and Murrell, 1988; Mallard et al., 1989). Very recently, based on the genome-wide study, *DQB1* was identified with significant effects on

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Submitted Apr. 17, 2015; Revised Jul. 13, 2015; Accepted Aug. 12, 2015

the susceptibility to viral load of the porcine circovirus type 2b (PCV2b), representing 2.8% of the variation (Tosky et al., 2015). Furthermore, results indicated that reproductive performance and production traits were related with SLA polymorphism (Renard and Vaiman, 1989; Gautschi and Gaillard, 1990). In relation to xenotransplantation research, SLA allele- or haplotype-specific xenogenic T-cell response are observed in human to porcine xenografts (Xu et al., 1999; Yamada et al., 2014).

Until now, many breeds of pigs were used for the characterization of SLA alleles and haplotypes. About 30 years ago, National Institutes of Health (NIH, USA) established MHC inbred miniature pig lines (Sachs et al., 1976) that are widely used in xenotransplantation research. Since then, 2 research groups have developed MHC inbred pig lines, i.e., Westran and Yucatan miniature pigs that can be used for xenotransplantation research (Lee et al., 2005; Smith et al., 2005a). Subsequently, SLA alleles were investigated in Clawn miniature pigs by the Japanese group using polymerase chain reaction-sequence specific primer (PCR-SSP) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (Ando et al., 2005). The same Japanese group identified SLA alleles and haplotypes in Microminipig (Ando et al., 2014). Therefore, it is clear that the development of SLA homozygous pig lines is very important in the future use of these experimental animals, especially for the biomedical research.

In order to use the Yucatan miniature pigs, we initially investigated 6 SLA loci in this pig population. The results of our study provide general guidelines not only for breeding of SLA homozygous Yucatan miniature pig lines but also for their use in transplantation and immunology-related research.

## MATERIALS AND METHODS

### Animals

Ear tissue samples from 114 Yucatan miniature pigs were obtained from Optifarm solution Inc. (Ochang, Korea). Genomic DNAs were extracted using PrimePrep genomic DNA isolation Kit (GeNetbio, Daejeon, Korea). The concentration of the genomic DNA was measured using NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA) at a wavelength of 260 nm and stored at  $-20^{\circ}\text{C}$  until use.

### Analysis of SLA haplotypes by PCR-SSP method

We used the PCR-SSP (sequence-specific primer) method for discriminating SLA alleles and haplotypes in Yucatan miniature pigs. This is a simple, fast and accurate genotyping method for SLA allele study. The SLA allele genotyping comprises 47 primer pairs for SLA class I alleles

(*SLA-1*, *SLA-3*, *SLA-2*), 47 primer pairs for SLA class II alleles (*DRB1*, *DQB1*, *DQA*) and positive control primers for the porcine  $\alpha$ -actin (*ACTA1* gene) (Supplementary Table 1) (Ho et al., 2009a; 2010). The PCR primers for the SLA class I alleles are presented in Supplementary Table 1. The class II primers are the same as used in Ho et al. (2010). The PCR amplification was carried out under the condition of denaturation for 10 min at  $94^{\circ}\text{C}$  followed by 35 cycles of  $96^{\circ}\text{C}$  for 15 s,  $65^{\circ}\text{C}$  for 20 s and  $72^{\circ}\text{C}$  for 20 s, with final extension step of 3 min at  $72^{\circ}\text{C}$ , and stored at  $4^{\circ}\text{C}$  using a 96-well GeneAmp PCR System 2700 (Applied Biosystems, Waltham, MA, USA). Each PCR reaction contained 25 ng of genomic DNA from Yucatan miniature pig,  $10\times$  PCR gold buffer (Applied Biosystems, USA) containing 25 mM  $\text{MgCl}_2$  (Applied Biosystems, USA), 2.5 mM of each dNTPs (GeNetbio, Korea), 10 pmol of forward and reverse primer pairs,  $1\times$  Cresol Red loading buffer (1.0 mM Cresol Red in 1,750 mM sucrose), 1.5 Unit of AmpliTaq Gold polymerase (Applied Biosystems, USA), 0.5 pmol porcine positive control primers designed from  $\alpha$ -actin gene (*ACTA1*) and 0.5  $\mu\text{g}$  of acetylated BSA (Promega, Madison, WI, USA) in a total volume of 5  $\mu\text{L}$ . Negative control was included to detect reagent contamination in each genotyping reaction. The PCR products were confirmed by 2.0% standard  $1\times$  TAE (Tris-acetate-EDTA) agarose gel electrophoresis at 150 V for 5 min using Micro SSP Gel System (One Lambda, Canoga Park, CA, USA).

### Lab on a chip analysis

The Agilent 2100 analyzer (Agilent Technologies, Palo Alto, CA, USA) was also used for known allele genotyping using the size differences. The microchip (LabChip 7500; Caliper Technologies, Mountain View, CA, USA) for the analyzer can be used for up to 12 samples and the results are obtained within 30 min. The Agilent 2100 bioanalyzer uses capillary-based electrophoresis on a microchip resulting in efficient and rapid size identification of small DNA fragments.

## RESULTS

### SLA alleles and haplotypes in Yucatan miniature pigs

SLA class I (*SLA-1*, *SLA-3*, and *SLA-2*) and SLA class II (*DRB1*, *DQB1*, and *DQA*) haplotypes of Yucatan miniature pigs were identified using low-resolution PCR-SSP and allele-specific primers as previously described (Ho et al., 2009b). In this study, SLA class I haplotypes (Lr-4.0, Lr-6.0, and Lr-0.25) and SLA class II haplotypes (Lr-0.5, Lr-0.7, and Lr-0.25) were identified in the 114 Yucatan miniature pigs (Supplementary Figures 1 and 2). Based on the SLA haplotypes identified in the 114 Yucatan miniature pig population, 5 SLA class I haplotypes, comprising 2

**Table 1.** SLA class I and II haplotypes and alleles identified in Yucatan miniature pig using a low-resolution PCR-SSP

Haplotype	Allele specificity								Haplotype frequency (%)
	SLA class I haplotype	SLA-1	SLA-3	SLA-2	SLA class II haplotype	DRB1	DQB1	DQA	Total
Lr-4.5 homozygote	Lr-4.0	04XX	04XX	04XX	Lr-0.5	05XX	02XX	02XX	17.54 (20/114)
Lr-6.7/6.7 homozygote	Lr-6.0	08XX	06XX	05XX	Lr-0.7	06XX	03XX	01XX	17.54 (20/114)
Lr-4.5/6.7 heterozygote	Lr-4.0	04XX	04XX	04XX	Lr-0.5	05XX	02XX	02XX	49.12 (56/114)
	Lr-6.0	08XX	06XX	05XX	Lr-0.7	06XX	03XX	01XX	
Lr-4.5/25.25 heterozygote	Lr-4.0	04XX	04XX	04XX	Lr-0.5	05XX	02XX	02XX	7.02 (8/114)
	Lr-25.0	11XX	03XX	07XX	Lr-0.25	13XX	09XX	04XX+w05XX	
Lr-6.7/25.25 heterozygote	Lr-6.0	08XX	06XX	05XX	Lr-0.7	06XX	03XX	01XX	7.02 (8/114)
	Lr-25.0	11XX	03XX	07XX	Lr-0.25	13XX	09XX	04XX+w05XX	
Lr-4.5/4.7 heterozygote	Lr-4.0	04XX	04XX	04XX	Lr-0.5	05XX	02XX	02XX	1.75 (2/114)
	Lr-4.0	04XX	04XX	04XX	Lr-0.7	06XX	03XX	01XX	

SLA, swine leukocyte antigen; PCR-SSP, polymerase chain reaction-sequence specific primer.

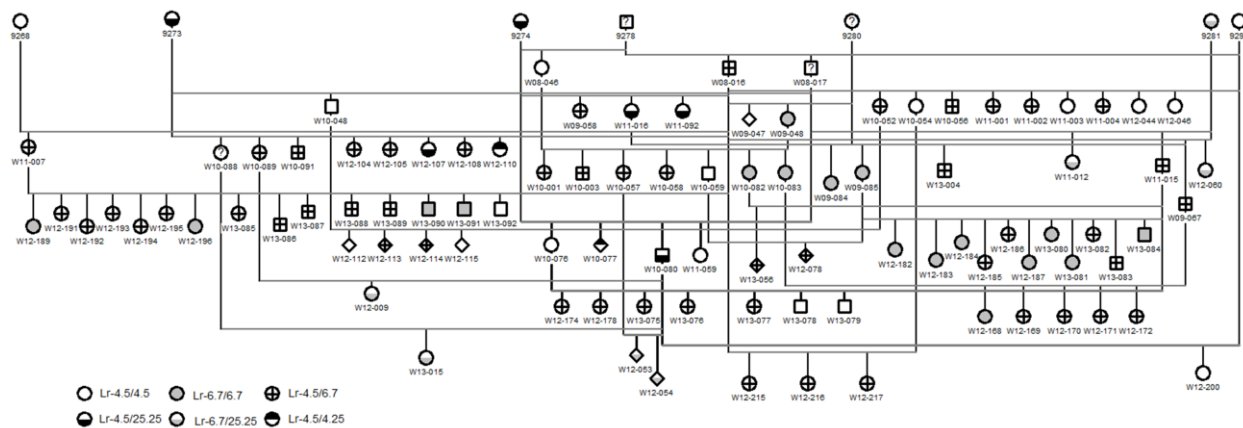
homozygous haplotypes (Lr-4.0/4.0 and Lr-6.0/6.0) and 3 heterozygous haplotypes (Lr-4.0/6.0, Lr-4.0/25.0, and Lr-6.0/25.0) were identified. Also, 5 SLA class II haplotypes, comprising 2 homozygous haplotypes (Lr-0.5/0.5 and Lr-0.7/0.7) and 3 heterozygous haplotypes (Lr-0.5/0.7, Lr-0.5/0.25, and Lr-0.7/0.25), respectively, were identified. The combination of SLA class I and class II haplotypes indicated that 2 homozygous haplotypes (Lr-4.5/4.5 and Lr-6.7/6.7) and 4 heterozygous haplotypes (Lr-4.5/6.7, Lr-4.5/25.25, Lr-6.7/25.25, and Lr-4.5/4.7), respectively, were investigated in this population (Table 1). Based on the combination of SLA class I and class II haplotype, Lr-4.5/6.7 had the highest frequency of 56%. Two homozygous haplotypes (Lr-4.5/4.5 and Lr-6.7/6.7) were the second highest frequency of 17.54% (Table 1). The pedigree information indicating the whole Yucatan miniature pigs were illustrated and their SLA class I and class II haplotypes were also presented (Figure 1).

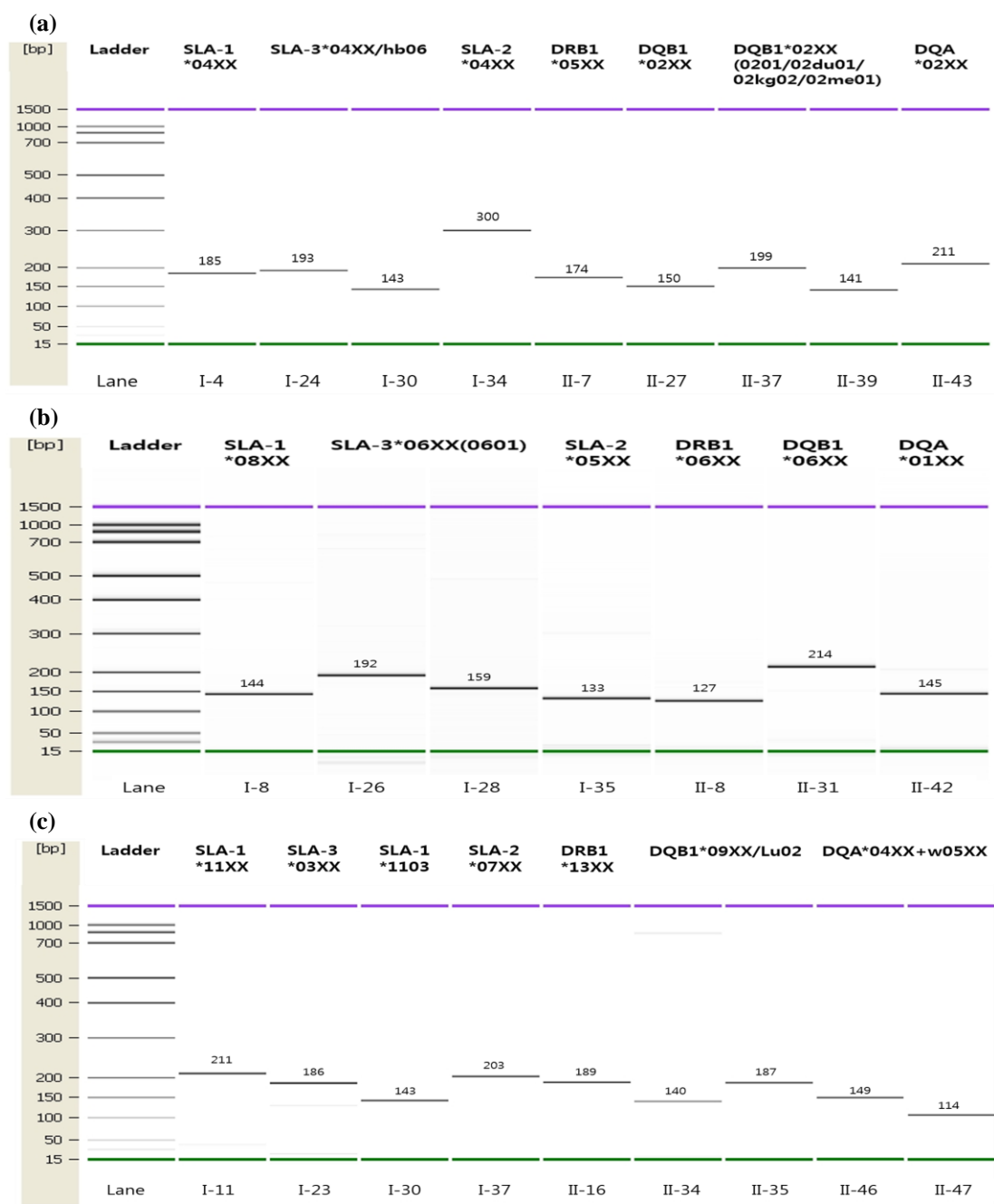
#### Genotyping of the SLA alleles using Agilent 2100 DNA

#### analyzer

Agilent 2100 DNA analyzer was applied for the efficient and fast genotyping of the SLA alleles identified in this study (Figure 2). Since only a small number of haplotypes was identified in the Korean Yucatan pig population, not all of the primers pairs were needed for allele identification. Therefore, only a sub set of primer pairs were applied in this study. The number of minimum markers for Lr-4.5 was 4 and 5 for class I and Class II genes, respectively. Also, 4 and 3 for class I and Class II genes, respectively, are needed for the genotyping of the Lr-6.7 haplotype. In case of genotyping Lr-25.25, 4, and 5 for class I and Class II genes, respectively, were needed. Altogether, 12 primer pairs and 13 primer pairs were required for the genotyping of class I and class II genes, respectively, for genotyping entire Yucatan miniature pigs.

During the experimental procedures, the observed product sizes did not exactly match the expected PCR product size. For example, the expected PCR product size for *SLA-I\*04XX* was 181 bp. However, we observed 185 bp

**Figure 1.** Pedigree of the Yucatan miniature pig population in Korea.



**Figure 2.** Results of the SLA genotypes for the Yucatan miniature pigs using the Agilent 2100 DNA analyzer. Information of the lane numbers is the locus specific class I and class II products that were shown in Ho et al. (2009a, b). The genotypes for each haplotype are represented (a) PCR results for haplotype 4.5, (b) PCR results for haplotype 6.7, (c) PCR results for haplotype 25.25. The numbers above the PCR bands are the product sizes. SLA, swine leukocyte antigen; PCR, polymerase chain reaction.

with the data from Agilent 2100 DNA analyzer. The size of the PCR products could be confirmed with the cloning and sequencing. The bp differences were actually due to the errors from the machine because the product size was estimated from the ladder used in the machine. Despite a few bp difference between the estimated and actual PCR product size, the results did not affect the allele identification in this study.

### DISCUSSION

Previously, Smith et al. (2005a, b) identified 4 SLA class I haplotypes (Lr-4.0, Lr-5.0, Lr-6.0, and Lr-7.0) and 4 SLA class II haplotypes (Lr-0.5, Lr-0.6, Lr-0.7, and Lr-0.8a) in Yucatan miniature pigs in USA. Haplotype Lr-25.25 was previously identified in LLC-PK1 cell line, which originated from the Hampshire breed. Therefore, in order to confirm the new haplotype, Lr-25.25, the PCR fragment for the alleles of

Lr-25.25 were directly sequenced and the results were aligned with reference sequences in the IPD-MHC database (<https://www.ebi.ac.uk/ipd/mhc/>). The results indicated that the sequences obtained are 100% identical with the alleles of Lr-25.25 (data not shown). Based on the haplotype analysis, Lr-25.25 homozygous animals do not currently exist in the Yucatan immature pig population in Korea.

Previously, RFLP (Chardon et al., 1985), PCR-RFLP (Fang et al., 2005) and genomic sequence-based typing (GBST) (Park et al., 2010; Thong et al., 2011), cDNA sequence-based typing (SBT) methods were applied for the SLA genotyping. Among these, the cDNA SBT typing method is a very accurate and direct method for defining SLA alleles. However, it requires more complicated experimental procedures such as cloning and sequencing of the PCR products (Ho et al., 2009a, b). In this study, a new method using Agilent 2100 DNA analyzer was developed, which is very efficient for the allele identification of a closed population with known pedigree information.

Primates are one of the best xenotransplantation donors based on the phylogenetic relationships. However, primates can share infectious diseases and also present ethical issues. Furthermore, there is a very high cost involved in breeding primates. The alternative species for the biomedical model animal is the pig due to the high reproductive performance, breeding in disease free environment and relatively less ethical issues, as compared with primates. The transgenic pigs, such as  $\alpha$ 1,3-galactosyltransferase gene knockout pigs, were used for the xenotransplantation research. Until now, porcine heart, kidney, liver, lung, and islets of Langerhans were the specific target organs for xenotransplantation research. Among these, islets of Langerhans have been clinically used in Mexico, Russia and China (Michel et al., 2015).

In conclusion, we have characterized 2 homozygous (Lr-4.5/4.5 and Lr-6.7/6.7) and 4 heterozygous (Lr-4.5/6.7, Lr-4.5/25.25, Lr-6.7/25.25, and Lr-4.5/4.7) haplotypes identified in the Yucatan miniature pigs in Korea. The results of this study will enable the selection and breeding of genetically stable Yucatan miniature pigs for use in biomedical and xenotransplantation studies.

## CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

## ACKNOWLEDGMENTS

This work was supported by a grant from (No. 20100023352) National Research Foundation of Korea, Republic of Korea.

## REFERENCES

- Ando, A., M. Ota, M. Sada, Y. Katsuyama, R. Goto, A. Shigenari, H. Kawata, T. Anzai, T. Iwanaga, and Y. Miyoshi et al. 2005. Rapid assignment of the swine major histocompatibility complex (SLA) class I and II genotypes in Clawn miniature swine using PCR-SSP and PCR-RFLP methods. *Xenotransplantation* 12:121-126.
- Ando, A., N. Imaeda, S. Ohshima, A. Miyamoto, N. Kaneko, M. Takasu, T. Shiina, J. K. Kulski, H. Inoko, and H. Kitagawa. 2014. Characterization of swine leukocyte antigen alleles and haplotypes on a novel miniature pig line, microminipig. *Anim. Genet.* 45:791-798.
- Chardon, P., C. Renard, M. Kirszenbaum, C. Gefotin, D. Cohen, and M. Vaiman. 1985. Molecular genetic analyses of the major histocompatibility complex in pig families and recombinants. *Int. J. Immunogenet.* 12:139-149.
- Fang, M., M. Braunschweig, X. Hu, L. Hu, J. Feng, N. Li, and C. Wu. 2005. Genetic variation of exon 2 of SLA-DQB gene in chinese pigs. *Biochem. Genet.* 43:119-125.
- Gautschi, C. and C. Gaillard. 1990. Influence of major histocompatibility complex on reproduction and production traits in swine. *Anim. Genet.* 21:161-170.
- Ho, C. S., J. K. Lunney, A. Ando, C. Rogel-Gaillard, J. H. Lee, L. B. Schook, and D. M. Smith. 2009a. Nomenclature for factors of the SLA system, update 2008. *Tissue Antigens* 73:307-315.
- Ho, C. S., J. K. Lunney, M. H. Franzo-Romain, G. W. Martens, Y. J. Lee, J. H. Lee, M. Wysocki, R. R. Rowland, and D. M. Smith. 2009b. Molecular characterization of swine leucocyte antigen class I genes in outbred pig populations. *Anim. Genet.* 40:468-478.
- Ho, C. S., J. K. Lunney, J. H. Lee, M. H. Franzo-Romain, G. W. Martens, R. R. Rowland, and D. M. Smith. 2010. Molecular characterization of swine leucocyte antigen class II genes in outbred pig populations. *Anim. Genet.* 41:428-432.
- Lee, J. H., D. Simond, W. J. Hawthorne, S. N. Walters, A. T. Patel, D. M. Smith, P. J. O'Connell, and C. Moran. 2005. Characterization of the swine major histocompatibility complex alleles at eight loci in westran pigs. *Xenotransplantation* 12:303-307.
- Lunney, J. K. and K. D. Murrell. 1988. Immunogenetic analysis of *Trichinella spiralis* infections in swine. *Vet. Parasitol.* 29:179-193.
- Mallard, B., B. Wilkie, and B. Kennedy. 1989. Genetic and other effects on antibody and cell mediated immune response in swine leucocyte antigen (SLA)-defined miniature pigs. *Anim. Genet.* 20:167-178.
- Michel, S. G., M. L. L. Madariaga, V. Villani, and K. Shanmugarajah. 2015. Current progress in xenotransplantation and organ bioengineering. *Int. J. Surg.* 13:239-244.
- Naziruddin, B., S. Durriya, D. Phelan, B. F. Duffy, B. Olack, D. Smith, T. Howard, and T. Mohanakumar. 1998. HLA antibodies present in the sera of sensitized patients awaiting renal transplant are also reactive to swine leukocyte antigens 1, 2. *Transplantation* 66:1074-1080.
- Park, K., H. Choi, L. M. Thong, O. J. Kwon, J. H. Kim, H. T. Lee, Y. B. Kim, S. B. Park, and C. Park. 2010. Simple and comprehensive SLA-DQB1 genotyping using genomic PCR

- and direct sequencing. *Tissue antigens* 76:301-310.
- Renard, C. and M. Vaiman. 1989. Possible relationships between SLA and porcine reproduction. *Reprod. Nutr. Dev.* 29:569-576.
- Sachs, D. H., G. Leight, J. Cone, S. Schwarz, L. Stuart, and S. Rosenberg. 1976. Transplantation in miniature swine: I. Fixation of the major histocompatibility complex. *Transplantation* 22:559-567.
- Shishido, S., B. Naziruddin, T. Howard, and T. Mohanakumar. 1997. Recognition of porcine major histocompatibility complex class I antigens by human CD8+ cytolytic T cell clones. *Transplantation* 64:340-346.
- Smith, D., J. Lunney, G. Martens, A. Ando, J. H. Lee, C. S. Ho, L. Schook, C. Renard, and P. Chardon. 2005a. Nomenclature for factors of the SLA class-I system, 2004. *Tissue Antigens* 65:136-149.
- Smith, D. M., J. K. Lunney, C. S. Ho, G. W. Martens, A. Ando, J. H. Lee, L. Schook, C. Renard, and P. Chardon. 2005b. Nomenclature for factors of the swine leukocyte antigen class II system, 2005. *Tissue Antigens* 66:623-639.
- Thong, L. M., H. Choi, O. J. Kwon, J. H. Kim, Y. B. Kim, J. W. Oh, K. Seo, S. C. Yeom, W. J. Lee, and C. Park. 2011. Systematic analysis of swine leukocyte antigen-DRB1 nucleotide polymorphisms using genomic DNA-based high-resolution genotyping and identification of new alleles. *Tissue Antigens* 77:572-583.
- Tosky, E, T. Engle, E. Davis, G. S. Plastow, S. Premaraj, S. D. kachman, and D. C. Ciobanu. 2015, Swine leukocyte antigen class II Region is associated with variation in Porcine Circovirus 2b susceptibility. In: *Proceedings of the Plant & Animal Genome XXIII*, San Diego, CA, USA. 234 p.
- Xu, X.-C., B. Naziruddin, H. Sasaki, D. M. Smith, and T. Mohanakumar. 1999. Allele-specific and peptide-dependent recognition of swine leukocyte antigen class I by human cytotoxic T-cell clones. *Transplantation* 68:473-479.
- Yamada, K., M. Tasaki, M. Sekijima, R. A. Wilkinson, V. Villani, S. G. Moran, T. A. Cormack, I. M. Hanekamp, J. S. Arn, J. A. Fishman, A. Shimizu, and D. H. Sachs. 2014. Porcine cytomegalovirus infection is associated with early rejection of kidney grafts in a pig to baboon xenotransplantation model. *Transplantation* 98:411-418.