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Molecular characterization of swine leukocyte antigen (SLA) gene diversity in Göttingen Minipigs

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1 INTRODUCTION

1.1 The Major Histocompatibility Complex (MHC)

The Major Histocompatibility Complex (MHC) is a cluster of genes that play an important role in immunological processes. This genomic region encodes glycoproteins that are expressed on the cell surface. The MHC molecules bind foreign peptide antigens or peptide antigens specific to the organism, which are then presented to the appropriate T cells. This leads to a cascade of immune responses (*Kulski et al. 2002, Wieczorek et al. 2017*).

The MHC molecules are mainly classified into two major classes of molecules, which differ not only in structure but also in function regarding the immune system. The MHC class I gene products are primarily associated with intracellular pathogens. On the other hand, the MHC class II molecules are associated with antigen presentation of extracellular pathogens (*Spurgin et al. 2010*). They are also expressed on different cells, but both cannot be found on red blood cells for their missing nucleus, therefore it is convenient for some specific pathogens to attack the erythrocytes and not get detected by e.g. cytolytic T cells.

The molecules encoded in the MHC specifically bind only peptides that fit into their binding pockets.

The MHC gene cluster in humans, so called Human Leukocyte Antigen (HLA), is located on chromosome 6 and contains over 200 genes. These genes are associated with many different diseases. The MHC class II genes may be associated with Kawasaki disease, Systemic Lupus, Asthma, Nephropathy or Leukemia, on the other hand class I genes are linked to diseases such as Leprosy, Type-1 autoimmune hepatitis or resistance to Malaria. Both MHC classes contribute to immunological processes in Multiple Sclerosis or Psoriasis *(Lokki et al. 2018, Fiorillo et al. 2017, Karnes et al. 2017, Yin Xianyong et al. 2015, Slager et al. 2013, Shrestha et al. 2016, Garamszegi et al. 2014)*.

The MHC region is polygenic, thus it carries several different genes of both MHC gene classes, so that each of the individuals is provided with a set of MHC molecules with different regions of peptide-binding properties. They are highly polymorphic, which means that the type of molecule occurs in numerous different versions within the population. The MHC genes are known to be the most polymorphic genes and their polymorphism has an intrusive influence on antigen recognition by T cells (*Sommer et al. 2005*).

The major histocompatibility complex is also partly involved in other processes such as shaping of mating preferences resulting in offspring MHC-heterozygosity *(Roberts et al. 2009)*.

1.1.1 MHC class I molecules

The MHC class I molecules are expressed on the surface of all nucleus-containing cells of the organism. They are heterodimers comprised of an alpha heavy chain with 3 subunits and a beta-2 (β2) microglobulin, whereby only the alpha component of the molecule is polymorphic. The β2-microglobulin is a 12 kDa polypeptide and is not encoded by the MHC. The complete MHC class I molecule has an immunoglobulin (Ig) fold structure and is non-covalently associated with β2-microglobulin. The α3 domain partially spans the plasma membrane and carries the CD8 binding site. The binding groove of the MHC class I molecule is formed by domains α1 and α2, and form a socket like opening, which can bind peptides of size 8-10 amino acids due to conserved tyrosine residues in the molecule (*Williams et al 2002, Wieczorek et al. 2017*). The antigen fragments are bound with strong hydrogen bonds in the binding pocket. In the middle of the groove there is a deep polymorphic pocket, which provides chemical and structural complementarity for one of the anchor residues of the peptide and thus significantly contributes to the allele-specific peptide binding (*Matsumura M. et al. 1992, Liepe et al. 2016*).

The MHC class I molecules are located on the lumen of the endoplasmic reticulum and bind cytosolic peptide fragments from the cell that originate from viral proteins or tumor proteins. The bound peptide fragments are further presented on the cell surface and recognized selectively by cytotoxic $CD8^+$ T cells. Tapasin-molecules, which catalyzes the loading of peptides, provides an effective antigen presentation (*Williams et al. 2002, Brocke et al. 2002, Sadegh-Nasseri et al. 2008, Dong et al. 2009*). The three different MHC class I molecules (HLA-A, HLA-B and HLA-C) are coded by eight exons with most polymorphic exons 2, 3 and 4 (*Lokki et al. 2018*).

1.1.2 MHC class II molecules

The MHC class II molecules have a common feature with the structure of MHC class I molecules. Like MHC class I molecules, MHC class II molecules are heterodimers. In this case, the MHC class II molecules consist of two noncovalent homogeneous peptides, alpha (e.g. DRA) and beta (e.g. DRB) chains, which have both extracellular and membrane spanning regions. There is no β2-microglobulin in their structure. Both chains of the MHC class II molecules are encoded by the MHC. The antigen binding cleft of the MHC class II molecules is constructed by the α1 and β1 domains.

In both, alpha and beta chain of class II proteins, there are Ig domains. The β2 domain of the MHC II protein carries the CD4 binding site.

The MHC class II glycoproteins are expressed on the surface of specialized antigen-presenting cells. They can be found on dendritic cells (DCs), macrophages and B lymphocytes. The antigens, which are mostly of extracellular origin and are internalized into the cell, bind to MHC II proteins and are presented then on the cell surface. The presented peptide is recognized by $CD4^+$ T cells. The contact of the $CD4^+$ T cells with the presented antigen triggers their activation, which subsequently causes the release of cytokines (*Lokki et al. 2018, Wiezcorek et al. 2017, Pos et al. 2013*).

1.1.3 The variability of the MHC

In the matter of evolution and organism's adaptation to different environmental influences, the processes can be exclusively characterized by the genetic modification. The high genetic variability of the MHC plays particularly an essential role in the adaptation of an organism to susceptibility to infectious or autoimmune diseases, or resistance to pathogens, thus ensuring the survival of the organism. For this evolutionary demand the ultimate polymorphism arose. In humans, 25,756 distinct HLA alleles have been reported (IPD-IMGT/HLA Release 3.38.0, 2019-10-17). The allelic variations differ between populations, therefore determining their susceptibility to various diseases. Under the most polymorphic HLA class I molecules are HLA-A genes with 5,735 reported alleles, HLA-B genes with 7,053 different alleles and HLA-C with 5,653 reported alleles. To HLA class II genes with high polymorphism rate belong HLA-DRB genes, HLA-DQB1 genes and HLA-DPB1 genes with 3,296, 1,771 and 1,519 reported alleles, respectively. According to Lokki et al., 10,000 different HLA haplotypes were recognized.

Polymorphism of the MHC molecules, and thus the allelic variation has an impact on the composition of the antigen-binding region of the molecules. With particularly diverse sequences of MHC genes, binding sites of different MHC molecules are competent to bind a broad spectrum of pathogen-derived peptides and present them to an appropriate T cell population. Several studies have discussed the advantage of heterozygosity of an individual in which the organism can bind, present and subsequently recognize by T cells a greater variety of antigen fragments, which give the organism a better reaction to peptides of pathogenic origin.

The variation that originates from genetic polymorphism, by altering the amino acid sequence of the molecule subunits, is narrowed to the amino terminal domains of the molecule that form the binding groove (*Hedrick et al. 2002, Sommer et al. 2005, Spurgin et al. 2010, Lokki et al. 2018*).

1.1.4 The MHC interaction with T cells

Adaptive immune system of vertebrates with two types of antigen receptors has two distinct ways of antigen detection. In contrast to B cells, T cells need antigens presented on the cell surface by MHC molecules. Different types of pathogens initiate distinct immunological responses that are kicked off by binding peptide fragments of foreign origin to MHC molecules. Originates the pathogen attacking the organism intracellularly, the peptide fragments are transported from cytosol -where they have been processed- to endoplasmic reticulum (ER) and bind to MHC class I glycoproteins. The peptide fragment, stabilizing the MHC molecule allows the molecule to be exported to the cell surface.

Extracellular foreign antigens are internalized into intracellular vesicles, in which the acidification takes place that activates the degradation of foreign proteins to peptide fragments that can be subsequently bound to a newly synthesized MHC class II molecule, afterwards transported to the cell surface as a peptide-loaded MHC class II molecule. The peptide fragments presented on the cell surface are detected by appropriate T cells with their T-cell receptors (TCRs), which are constructed of two polypeptide chains bound through disulfide bonds. These TCRs interact with the peptide-loaded MHC molecules. For the transduction of the signal generated by the contact with the peptide-loaded MHC glycoproteins, CD3 molecules need to be present. The signals transduced with help of these molecules then activate the T cells that, in case of MHC class I molecule interacting with $CD8^+$ T cells, initiate the response of cytotoxic T cells to eliminate the respective antigen-presenting cells. Supposing the $CD4^+$ T cells are bound to peptide-loaded MHC-II molecules, the transduced signal can start for several responses, guided by the respective cytokine milieu. For instance, an Interleukin (IL) 12-producing DC is presenting the antigen, the $CD4^+$ T cell is activated and becomes an Interferon (IFN)-γ producing effector T cell. The IFN-γ producing effector T cells activate macrophages to kill intracellular pathogens (*Forman et al. 1984*, *Wieczorek et al. 2016, Call ME et al. 2002, Neefjes et al. 2011, Rock et al. 2016*).

1.2 The Swine Leukocyte Antigen (SLA) complex

The porcine MHC or Swine Leukocyte Antigen (SLA) complex is one of the densest regions of the porcine genome. It is divided in three regions: SLA class I, SLA class III, and SLA class II. The SLA genomic complex can be found on chromosome 7, with SLA class I and class III on the p (short) arm, and SLA class II on the q (long) arm of the chromosome. The human and swine leukocyte antigens were compared in numerous studies. The results identified some differences including nonappearance of some class I-like loci and lack of HLA-DP-like loci in

swine. Of all mammalian MHCs is the SLA the smallest one with the three regions covering 1.1 Mb, 0.7 Mb and 0.5 Mb, respectively (*Vaiman et al. 1998*, *Chardon et al. 1999, Lunney et al. 2009*). The genes coded by SLA are of the highest importance in concept of immunological responses to infectious agents and vaccines. Alongside their fundamental function of protecting the organism against pathogens, the SLA showed linkage to growth rate and fat accumulation in various complex studies (*Renard et al. 2006, Ho et al. 2009*).

SLA molecules are expressed co-dominantly, meaning that both inherited alleles are producing the resulting molecule.

Like Human Leukocyte Antigen glycoproteins, the SLA molecules operate in inspecting and presenting processed antigens to T cells.

1.2.1 Genomic structure and function of the SLA-class I genes

The SLA class I genes cover eight exons, of which the exons 2-4 encode the three alpha-domains that form the variable component of the SLA class I molecule. The molecules transmembrane domain is encoded by the exon 5 and the cytosolic domain is encrypted in exons 6, 7 and 8 (*Lunney et al. 2009*). The assembly of SLA class I genes include three classical genes, intituled SLA-1, SLA-2 and SLA-3, that besides encoded regular functions carry also promoter coding regions. Further, the gene cluster contains several pseudogenes such as SLA-4 or SLA-9, and three non-classical genes, the SLA-6, SLA-7, SLA-8 (*Renard et. al 2001, Smith et. al 2005, Tennant et al. 2007*). The classical class I genes are eminently polymorphic, of which the SLA-1 and SLA-2 have the highest expression level (*Zhang et al. 2011, Pedersen et al. 2014, Gao et al. 2017*). The highest level of the SLA class I molecules is concentrated in the antigen binding site that is created by the alpha-1 and alpha-2 domains. All three classical class I loci encode functional genes that are pertinent for the activation of $CD8^+$ T lymphocytes (*Sørensen et al. 2017*).

Like human MHC class I glycoproteins, the SLA class I molecules are composed of two membrane-distal domains alpha-1 and alpha-2 that assemle a peptide-binding cleft, one membrane-proximal domain alpha-3 and a transmembrane section and a cytosolic tail. The alpha-3 domain represents a binding site for CD8 co-receptor. The complete alpha chain is non-covalently associated with a β2 microglobulin. These molecules are expressed on the surface of all cells containing a nucleus. Peptide fragments originated from cytosol during a viral infection or tumorous cell transformation are presented to and recognized by $CD8^+$ T cells that develop to cytotoxic T cells which are able to kill cells presenting the respective antigens (*Sullivan et al. 1997, Wieczorek et al. 2017*).

1.2.2 Genomic structure and function of the SLA-class II genes

SLA class II are genes encoded by porcine MHC are primarily expressed on the surface of antigen presenting cells (APC) and are composed of two non-covalently bound chains, an α and a β chain. After binding of mostly exogenous antigen fragments in their peptide-binding cleft consisting of α1 and β1 domains, they present the peptides derived from exogeneous antigens to $CD4^+$ T cells. The SLA class II region is separated from region III by the centromere. There are two groups of genes encoded in the SLA class II region: SLA-DR and SLA-DQ. With the leader sequence encoded by exon 1, the SLA class II genes encoding for the α1 and α2 domains are encrypted in exon 2 and 3, respectively. Both DRA and DQA genes consist also of a fourth exon encoding the transmembrane and cytoplasmatic domains. The SLA class II genes accountable for the structure of the β-chain have similar molecular constitution with exception of DRB1 and DQB1 genes. As other β-chain genes they have a leader sequence encoded in exon 1, extracellular domains in exons 2 and 3, but their transmembrane domain is encrypted by exon 4 and a supplementary exon 5 of DQB1 and exons 5 and 6 of DRB1 encode for their cytoplasmic domains. These genes show high homology with their analogues in humans (*Lunney et al. 2009, Ho et al. 2010, Piriou-Guzylack & Salmon et al. 2008*). SLA-DRB1 and SLA-DQB1 represent highly polymorphic loci. SLA-DQA locus shows only an average polymorphism, while the polymorphism of the SLA-DRA is rather restricted.

1.2.3 The variability of the porcine MHC

The genetic variability of porcine MHC as an adaptation element to environmental influences indicates the pig's susceptibility to infectious agents, thus its survival. The allelic variation in pig in comparison to humans is relatively low. Despite the low number of known SLA alleles, the amount of reported alleles is still increasing. The most polymorphic SLA class I molecules include SLA-1 genes with 90 reported alleles, SLA-2 genes with 96 reported alleles and SLA-3 with 41 different alleles. To the most important SLA class II polymorphic genes belong SLA-DRB1 genes, SLA-DQB1 genes and SLA-DQA genes with 99, 53 and 26 reported alleles (IPD-MHC Release 3.3.0.0 (2019-06-13) build 126) *(Maccari et al. 2017).*

1.3 Göttingen Minipigs as an animal model in biomedical research

The Göttingen Minipig (GMP) is the smallest domestic pig breed known in the world. Göttingen Minipigs or pigs in general are a solid model in biomedical, pharmaceutical and agronomic research including observation of pathogenesis of viral infections. Because of identified

similarities with humans at anatomical, physiological and immunological level, especially in the common features of the major histocompatibility complex, this animal model has become an important component in allo- and xenotransplantation studies (*Ho et al. 2006*, *Heckel et al. 2015, Schook et al. 2015*). With their small-sized body, adult body weight of 30-40 kg and their propriety for most testing purposes, Göttingen Minipigs are one of exceptionally suitable models in experiments focusing on immunological response and disease resistance.

As a crossbreeding result of Minnesota miniature swine, German Landrace pig and Vietnamese potbelly pig, the Göttingen Minipig was produced in Germany at the University of Göttingen with the goal of developing a fitting model for biomedical research with a determined genetically background. This pig model became accessible in the late 1960's and was initially developed for studying dermal and chronic diseases. Their similar anatomical and functional constitution to human, as well as their metabolic resemblance, led the Göttingen Minipig to a popular position in other areas of biomedical and pharmaceutical research. Nowadays, numerous fields of biomedical research are benefitting from this pig model (*Figueiredo et al. 2019, Wakeman et al. 2006, Bollen et al. 1997*).

1.4 SLA Typing methods

Typing methods for the MHC region in swine are relevant for improvement of breeding strategies, and by additional identification of T-cell epitopes also for an enhancement in the development of new vaccines, thus leading to an effective prophylaxis. Therefore, the typing methods with precise and responsive outcome are of a critical importance. A very effective way to molecularly characterize the porcine MHC with low costs are low-resolution typing methods such as PCR with sequence-specific primers (PCR-SSP), microsatellite (MS) markers or PCR-restriction fragment length polymorphism (PCR-RFLP). The PCR-SSP principle lies in amplification of only perfectly matched DNA with specific primers. Analysis of the SLA with application of MS markers presents a quick typing method but its resolution is conditioned by markers present in the MHC. SLA Typing using PCR-RFLP can be applied to analyze allelic divergences. Upon these methods, there are more time-consuming high-resolution strategies that are sequence-based. Despite its slow process, it offers much more accurate results (*Essler et al. 2013, Gimsa et al. 2017, Lunney et al. 2009, Ho et al. 2009*).

1.4.1 Low-resolution typing (PCR-SSP assay)

Low-resolution typing acquired by PCR using sequence-specific primers represents a time-efficient approach for typing porcine MHC with relatively low expense, but yet as a valuable

information source (*Olerup et al. 1993, Ho et al. 2009*). SLA Typing using the PCR-SSP assay relies on genomic DNA and therefore supports a rapid evaluation of numerous animals. This method provides an exceptionally convenient technique for precise determination of SLA class I and class II alleles embedded in porcine MHC. Its rising recognition is based upon its precision and effectiveness but the higher demand on this method reposes on its accessible reproducibility.

1.4.2 High-resolution typing (Sequence-based typing, SBT)

Since there is a high interest in tumor, virus infection and transplantation studies and strong involvement in enhancing of vaccines, thus investigation of immunological responses at the peptide level, the relevance of precise molecular characterization of the porcine MHC is increasing. High-resolution typing, by making use of sequence-based typing (SBT) strategies, resolves this strong demand at a very accurate degree. Sequence-based typing methods allow the determination of expressed SLA class I and class II alleles, thus an assignment of haplotypes, along with uncovering potential novel haplotypes in various pig breeds (*Luetkemeier et al. 2009, Sørensen et al. 2017, Le et al. 2012*).

1.5 Aim of the study

Since 2017, the Institute of Immunology at the University of Veterinary Medicine Vienna is collaborating with the Institute for Transfusion Medicine from the Hannover Medical School (MHH, Germany) on SLA-typing mismatch donor pairs of Göttingen Minipigs for allo-transplantation research. In previous studied cohorts of 30 pigs, we found evidence for potential novel SLA class I and SLA class II haplotypes together with a certain number of unknown ('blank') alleles. SLA genotyping data of the studied cohort also provide evidence for private alleles and haplotypes that are only present in the Göttingen Minipig population.

In the presented study, the investigated cohorts comprised 69 Göttingen Minipigs. These cohorts included animals that were chosen later for mismatch donor allo-transplantation in the ongoing research projects conducted at the MHH. For this reason, these Göttingen Minipigs needed to be characterized on the molecular level, to determine their SLA genotypes and haplotypes. To determine the SLA class I and SLA class II background in these minipigs, low resolution typing methods applying the PCR-SSP assay were used. By applying 96 allele-group specific primer pairs it was possible to reveal the SLA class I and SLA class II alleles together with the haplotypes occurring in the genome of Göttingen Minipigs. To determine unknown or novel alleles, high-resolution typing strategy could be applied.

The following underlying hypotheses have been addressed in this study:

(1) As a potential result of extensive breeding, Göttingen Minipigs show a reduced diversity in their swine leukocyte antigen (SLA) genes.
(2) The SLA background of Göttingen Minipigs might comprise private haplotypes.
(3) Accordingly, this pig line could harbor potential novel SLA haplotypes.

2 MATERIAL AND METHODS

2.1 Studied animals

In this project we analyzed 69 Göttingen Minipigs from the company Ellegaard, Dalmose, Denmark that were divided in five separate cohorts. Five Göttingen Minipigs belonged to Cohort Merck from 2016 of which three minipigs were females and two males, 12 Göttingen Minipigs belonged to Cohort Sanofi 2016 with unknown gender, and 52 minipigs were divided in Cohort 38, Cohort 39 and Cohort 40 with 16, 18 and 18 minipigs, respectively. Each of the latter cohorts were half males and half females. The five cohorts under investigation are listed in Table 1 (Tab. 1) along with the internal identification (ID) number for each minipig.

Tab. 1: Studied animals listed by cohort of origin.

Cohort	internal ID	Cohort	internal ID	Cohort	internal ID	Cohort	internal ID
Merck 2016	F01	Cohort 38	M01	Cohort 39	M01	Cohort 40	F01
	F02		M03		M02		F02
	F03		M06		M03		F03
	M01		M07		M04		F04
	M02		M08		M05		F05
			M15		M06		F06
Sanofi 2016	#01		M16		M07		F07
	#02		M19		M08		F08
	#03		F03		M09		F09
	#04		F06		F10		M10
	#05		F12		F11		M11
	#06		F13		F12		M12
	#07		F15		F13		M13
	#08		F16		F14		M14
	#09		F17		F15		M15
	#10		F18		F16		M16
	#11				F17		M17
	#12				F18		M18

Male minipigs are displayed in light-green color, females in light-orange color; Minipigs with unknown gender are left uncolored. The 69 Göttingen Minipigs are divided in cohorts based on their origin. ID = identification number.

Whole blood of altogether 69 Göttingen Minipigs was received from Ellegaard (Dalmose, Denmark) and subjected to DNA extraction. Combined genotypes and haplotypes of SLA class I (SLA-1, SLA-2 and SLA-3) and class II (SLA-DRB1, SLA-DQB1 and SLA-DQA) allele groups of all (Göttingen) Minipigs were established by low resolution SLA-typing method using PCR-sequence-specific primers (SSP).

2.2 Low resolution typing (PCR-SSP)

2.2.1 DNA extraction

Genomic DNA (gDNA) was extracted from blood samples using the E.Z.N.A Blood DNA Mini Kit (Omega Bio-tek, Inc., Nocross, GA, USA) that provides a rapid method for the isolation of genomic DNA applicable in downstream PCR assays. The whole blood samples were incubated 1:5 (v/v%) with EL Buffer (Qiagen, Hilden, Germany) for 15 minutes to lyse the erythrocytes, and then centrifuged at 400xg for 10 minutes at 4°C (Heraeus Multifuge 1S-R, Heraeus, Hanau, Germany). The supernatant containing the lysed erythrocytes was discarded completely. For the DNA extraction from pelleted leukocytes, 25 µl OB Protease Solution, 250 µl BL Buffer and 25 µl RNAse A (Sigma Aldrich, St. Louis, MO, USA) were added to the samples and incubated on a heating block at 65°C for 10 minutes. After incubation, 260 µl 96% Ethanol were pipetted to the samples and vortexed for 20 second at a maximum speed. The whole cell lysate was transferred to a HiBind DNA Mini Column inserted into 2 ml Collection Tube and centrifuged at 13,300 rpm for 1 minute (Biofuge Pico, Heraeus). Onto the HiBind DNA Mini Column 500 µl HBC Buffer and 2x 700 µl DNA Wash Buffer were added, centrifuged at 13,300 rpm after each step and the flow through was discarded. The empty HiBind DNA Mini Column was centrifuged at maximum speed for 2 minutes to remove residual traces of ethanol that may interfere with downstream applications (drying step). Next, the HiBind DNA Mini Columns were transferred into nuclease-free 1.5 ml microcentrifuge tubes. For eluting the gDNA, 150 µl Elution Buffer were heated to 65°C and added to the HiBind DNA Mini Column. After incubation of 5 minutes at room temperature (RT), they were centrifuged at 10,000 rpm for 1 minute (Biofuge Pico, Heraeus). Every sample was re-eluted, meaning, for every sample, the flow through was pipetted onto the HiBind DNA Mini Column and centrifuged again under the same conditions.

Afterwards, DNA concentration of all samples was measured with NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA) by reason of estimating the right amount of DNA samples that has to be pipetted into the PCR reactions and the quality of the DNA. To determine the

concentration of DNA, a microvolume sample size of 1.5 µl was pipetted directly onto the pedestal. The measurement of the DNA concentration is accomplished with UV-spectrometer at the nucleic acid absorption maximum of 260 nm. With altered equation based on the Lambert-Beer equation for computation of nucleic acid concentration, the DNA concentration was estimated. The ratios 260/280 and 260/230 determine the quality and purity of the extracted DNA. The purity of measured DNA is estimated by ratio of absorbance at 260 nm and 280 nm. A nucleic acid sample with a 260/280 nm ratio of 1.8-2 is considered as 'pure' DNA. If the ratio is considerably lower, it indicates a possible presence of contaminants such as proteins or phenol. The secondary measure of DNA purity of absorbance at 260 nm and 230 nm should show a ratio of 2 or more to be considered as 'pure'. If co-purified contaminants are present in DNA samples the 260/230 ratio is significantly lower.

2.2.2 α-Actin (ACTA1)

Before the minipigs were tested for which SLA class I and class II genes are present in their MHC region, a control PCR partially targeting α-Actin (ACTA1) was performed. ACTA1 PCR served as verification of the applicability of the DNA samples to further examination by PCR-SSP. A PCR reaction was set up mixing 1 µl 50x ACTA1 primer mix (*Ho et al. 2009*) with 5 µl 2x HotStar Taq DNA Polymerase Mix (Qiagen, Hilden, Germany), 1 µl Coral Load dye (Qiagen) and 3 µl DEPC-treated water. To the pre-mixed PCR reactions in 0.2 ml PCR tubes, 2 µl DNA of each minipig were added and amplified under cycling conditions as summarized in Tab. 2. For analyzing the PCR products by gel electrophoresis, a 1.5% agarose gel was used. Subsequent gel casting steps were performed as later described in section 2.2.4. with the exception of running a DNA ladder marker (FastRuler® Low Range DNA ladder, Thermo Fisher Scientific) alongside with the samples.

2.2.3 PCR-SSP assay

All positive animals were subjected to SLA Typing on a 96-well plate by using the PCR-SSP assay. Therefore, a typing plate was prepared with different optimized forward and reverse SLA class I and II typing primer pairs with a total volume of 1.5 µl per well. The list of primer pairs used in this project is provided in section 7.3. ACAT1 was used as an internal positive control with a known PCR product size of 516 base pairs (bp). In a total volume of 10 µl, 20 ng DNA should be used per well by adding the master mix containing 5 µl 2x HotStar Taq DNA Polymerase Mix (Qiagen), 1 µl 10x Coral Load dye (Qiagen), and 0.5 µl DEPC-treated water.

Using a multi dispenser pipette (Eppendorf, Hamburg, Germany), 9 µl of the master mix without DNA at positions H1 and H7, and with DNA were distributed to the 96 wells of the PCR plate. After ensuring by centrifugation that all PCR reactions were at the bottom of the wells, the thermal cycler was programmed according to cycling parameters summarized in Tab. 2. The PCR plates were sealed and placed in the thermal cycler (T Gradient thermal cycler; Biometra, Göttingen, Germany). The thermal cycling protocol contained an initial incubation at 95°C for 15 minutes, followed by 35 cycles of 30-second long intervals of temperature alternations with melting temperature at 95°C, annealing temperature at 60°C and extension temperature at 72°C, finished with a temperature decline to 15°C (*Ho et al. 2009, Ho et al. 2010*).

Tab. 2: PCR-SSP thermal cycling conditions.

Step		Temperature	Duration
Initial incubation		95°C	15 minutes
35 cycles	Melting	95°C	30 seconds
	Annealing	60°C	30 seconds
	Extension	72°C	30 seconds
Hold		15°C	indefinite

2.2.4 Agarose gel electrophoresis

To separate DNA fragments of varying sizes, agarose gel electrophoresis has been proven to be the most effective way. The DNA was loaded to pre-cast wells in the gel and a current was applied. Because of the negatively charged phosphate backbone of the DNA molecule, the DNA fragments migrated towards the positively charged anode. Identification of the PCR product size was done by running a DNA ladder marker with defined length of DNA fragments alongside with the samples to be analyzed. In detail, 3.6 g of electrophoresis-grade agarose (LE Agarose, Biozym Scientific, Oldendorf, Germany) was added to 120 ml of 1x TAE (TRIS/acetic acid/EDTA) electrophoresis buffer to obtain a 3% (w/v) agarose gel. The agarose was melted in a microwave oven. Afterwards, the melted agarose needed to be cooled to 55-65°C before it was mixed with 12 µl of 1:20 GelStar™ Nucleic Acid Gel Stain (Cambrex, Rockland, USA) to enable UV light visualization of the DNA fragments after the run. The melted agarose was poured into a prepared gel tray where 6 combs (each carrying 16 slots) have already been inserted. After complete hardening of the gel, the gel tray was placed into an electrophoresis chamber (EasyPhor® Horizontal Gel Electrophoresis units; Cleaver Scientific Ltd. via Biozym Scientific) filled with 1x TAE (TRIS/acetic acid/EDTA) electrophoresis buffer. After PCR, the entire reactions were pipetted to the gel slots, and electrophoresis was

performed for 2 minutes at 150 Volt and additional 15-20 minutes at 120 Volt (PowerPac® Basic, Bio-Rad Laboratories, Vienna, Austria) or until the positive control bands were well separated from the allele group-specific bands. After migration through the gel, the separated DNA fragments could be visualized with an UV transilluminator at 320 nm and a picture of the gel was taken with a digital camera (G: BOX, Syngene, Synoptics Ltd, Cambridge, England). The images were saved for later data evaluation.

2.2.5 Data evaluation and assignment to known haplotypes

The obtained images from low-resolution typing were evaluated with pre-made worksheet (provided in Tab. 3 and 4) containing potentially obtained allele groups and/ or alleles resulting from PCR with sequence-specific primers. For every primer pair there was a single allele or a group of alleles that could be present on the scanned sequence amplified by PCR, including the expected base pair lenght. In the images an ACTA1 band should be also visible that was used as an internal positive control and helped by identifying the base pair lenght of potentially positive bands from the estimated lenght of ACTA1 with approximately 516 bp (*Ho et al. 2009*). According to previous studies, the different allele groups were combined to assign the two haplotypes that were most likely present in a certain animal.

As an example, the low-resolution SLA class I genotype SLA-1*05XX, SLA-1*15XX, SLA-2*05XX, SLA-2*08XX, SLA-3*01XX, and SLA-3*03XX (GMP2.0), can be assigned to the haplotypes Lr-10.0 (SLA-1*05XX, SLA-2*08XX, SLA-3*03XX) and Lr-67.0mod (SLA-1*15XX, SLA-2*05XX, SLA-3*01XX).

Accordingly, the low-resolution SLA class II genotype SLA-DRB1*01XX, SLA-DRB1*03XX, SLA-DQB1*05XX, SLA-DQB1*03XX(03:01), SLA-DQA*04XX, and SLA-DQA*01XX (GMP0.1) can be assigned to the haplotypes Lr-0.21 (DRB1*01XX, DQB1*05XX, DQA*04XX) and Lr-0.03 (DRB1*03XX, DQB1*03XX(03:01), DQA*01XX).

The overall haplotype assignments were based on previously published data describing the allele group configuration of the different haplotypes and frequency of occurrence of alleles as haplotypes (*Ho et al. 2009, Ho et al. 2010, Essler et al. 2013, Pedersen et al. 2014, Gimsa et al. 2017, Hammer et al. 2020*).

Tab. 3: Evaluation worksheet for SLA class I

	H	G	F	E	D	C	B	A
1	Negative Control	209 bp P1023 SLA-1*01XX(all)	147 bp P1002 SLA-1*02XX(all)	181 bp P1011 SLA-1*04XX(all)	220 bp P1030D2 SLA-1*05XX(all); SLA-1*an02	163 bp P1072D5 SLA-1*06XX(all); SLA-1*1301	220 bp M1007 SLA-1*07XX(all)	138 bp P1039D3 SLA-1*08XX(all)
2	195 bp P1104D6 SLA-1*09XX(all)	180 bp M1002 SLA-1*w10XX(all)	182 bp P1182D1 SLA-1*11XX(all)	119 bp P1157 SLA-1*12XX(all)	211 bp P1055D3 SLA-1*13XX(all)	219 bp P1212D1 SLA-1*14XX(all)	253 bp P2079D2 SLA-1*15XX(all); SLA-1*es11; SLA-2*01XX(all)	173 bp P1049 SLA-1*16XX(all)
3	134 bp N2002 SLA-1*16XX(all); SLA-1*an02; SLA-2*03XX(all); SLA-2*es22	208 bp P1174D5 SLA-1*cs02	193 bp P1172D3 SLA-1*rh03; SLA-2*es22	130 bp P1206D1 SLA-1*rh03; SLA-1*st11	196 bp P1190D1 SLA-1*sk13	177 bp P3005D3 SLA-3*01XX(all)	183 bp P3030D3 SLA-3*03XX(all); SLA-3*08XX(all)	192 bp P3046BU1 SLA-3*04XX(all); SLA-3*hb06; SLA-2*15XX(all)
4	138 bp P3019D2 SLA-3*05XX(all)	187 bp P3127 SLA-3*06XX(all); SLA-3*07XX(all)	152 bp P3113U3 SLA-3*07XX(all)	152 bp P3057U2 SLA-3*0601	139 bp P3059D1 SLA-3*0602	139 bp P3116 SLA-1*1103; SLA-3*04XX(all); hb06; 03XX excluding 03pt31	172 bp P2003U2 SLA-2*01XX(all)	138 bp P2008D1 SLA-2*02XX(all)
5	89 bp P2009U1 SLA-2*03XX(all)	311 bp N2003D1 SLA-2*04XX(all)	127 bp P2017 SLA-2*05XX(all)	125 bp P2020U1 SLA-2*06XX(all)	199 bp P2077 SLA-2*07XX(all)	126 bp P2025D1 SLA-2*w08XX(all)	177 bp P2032D2 SLA-2*w09XX(all)	104 bp P2074 SLA-2*10XX(all)
6	123 bp P2041D2 SLA-2*11XX(all)	160 bp P2046 SLA-1*es11; SLA-2*12XX(all)	117 bp P2054U1 SLA-1*1103; SLA-2*w13XX(all)	131 bp P2174 SLA-1*09XX(all); SLA-2*w14XX(all); 16XX(all); jh02	90 bp P2137U1 SLA-2*15XX(all); SLA-2*es22	196 bp P2180 SLA-2*0601~02/06me01; w09an02/w09sn01; 16XX(all)	138 bp P2181D1 SLA-1*07XX(all); SLA-2*02XX(all) SLA-2*16XX(all); SLA-2*an04	175 bp P2170 SLA-2*jh02

The evaluation table for SLA class I consisted of 48 cells with estimated PCR product length, primer set ID and potential allele group or groups that could be obtained with each primer set. The coordinates were corresponding with typing plate coordinates. bp = base pairs

Tab. 4: Evaluation worksheet for SLA class II.

	H	G	F	E	D	C	B	A
7	Negative Control	162 bp PRB003 DRB1*01XX(all) ,	203 bp PRB098 DRB1*01XX(all) DRB1*be01/ha04/me02	115 bp PRB085 DRB1*02XX(all)	180 bp GR006 DRB1*03XX(all)	206 bp SR008D2 DRB1*04XX(all)	172 bp PRB016U2 DRB1*05XX(all)	122 bp PRB023D1 DRB1*06XX(all)
8	133 bp PRB026 DRB1*07XX(all)	108 bp PRB111 DRB1*08XX(all) DRB1*ka04/ka05/oj01	105 bp PRB0108 DRB1*09XX(all) DRB1*du05/La02/oj02	157 bp PRB034D3 DRB1*09XX(all) DRB1*ka09/kb02/La02/La04	135 bp PRB037U1 DRB1*10XX(all) DRB1*er01/La03	109 bp PRB042D2 DRB1*11XX(all)	186 bp PRB048D1 DRB1*w12XX(all)	182 bp GR016 DRB1*13XX(all)
9	113 bp PRB056 DRB1*14XX(all)	160 bp PRB107 DRB1*ka13	134 bp PRB053U1 DRB1*ka14	202 bp PRB092D1 DRB1*kb02/kb03N/kb04N	197 bp PRB090 DRB1*La03/La04/La05	117 bp PRB063U3 DRB1*0403~04 DRB1*1101/11ac21; DRB1*ka13	160 bp SR006U1 DRB1*0401~02/04ga01/04ta01	118 bp SR007 DRB1*0401~02/04ga01/04ta01
10	165 bp PQB012D4 DQB1*01XX(all)	180 bp PQB014 DQB1*01XX(all) DQB1*sh03	146 bp PQB015U2 DQB1*02XX(all)	166 bp PQB018U1 DQB1*03XX(all)	197 bp MQ005 DQB1*04XX(all)	193 bp PQB051D3 DQB1*05XX(all)	204 bp PQB092D1 DQB1*06XX(all) DQB1*zs12	154 bp PQB031U1 DQB1*07XX(all)
11	148 bp PQB009D9 DQB1*08XX(all) DQB1*zs13	146 bp PQB033D2 DQB1*09XX(all)	180 bp PQB035D4 DQB1*09XX(all) DQB1*Lu02	161 bp PQB087 DQB1*es51 DQB1*zs14	193 bp PQB052U3 DQB1*0201/0204/02du01/02kg02/02me01 DQB1*Lu02/zs13	176 bp GQ002D3 DQB1*0202/02zs16	133 bp PQB002U3 DQB1*0201/0203/02du01/02kg02/02me01	165 bp PQB044U1 DQB1*0203/02zs16 DQB1*0301
12	173 bp GQ003D7 DQB1*0202/0204 DQB1*zs13	141 bp PQA004D1 DQA*01XX(all)	21 0 bp PQA025U1 DQA*02XX(all) DQA*ka01	160 bp PQA009D3 DQA*03XX(all)	124 bp PQA014 DQA*04XX(all)	148 bp PQA013D2 DQA*04XX(all)	111 bp PQA019D2 DQA*w05XX(all)	120 bp PQA023D1 DQA*ka01

The evaluation table for SLA class II consisted of 48 cells with estimated PCR product length, primer set ID and potential allele group or groups that could be obtained with each primer set. The coordinates were corresponding with typing plate coordinates. bp = base pairs

2.3 High-resolution typing (Sequence-based typing)

For characterization of the MHC genes in swine a high-resolution typing method was used based on the sequencing of the wanted genetic information with possibility of more exact results, than in the previously conducted PCR-SSP assay. The most commonly used method in this context is the so-called sequence-based typing (SBT) approach.

Characterization of SLA haplotypes with potential novel haplotypes was discovered either by reverse transcription-polymerase chain reaction (RT-PCR)-based typing method or conventional endpoint PCR. In addition of running PCR reactions, both methods included cloning and sequencing of presumed/ unknown alleles.

This method was used for alleles that were not detected explicitly by low-resolution typing using the PCR-SSP approach. For the two minipigs #8 and #9 from Cohort Sanofi 2016, we had to prove whether or not these two animals are truly homozygous for SLA-DRB1 and SLA-DQB1 and which alleles they carry. For analyzing SLA-DRB1 and SLA-DQB1 by SBT, forward- and reverse-primers were used, which were designed for targeting genomic DNA. Therefore, we could make use of the previously isolated genomic DNA, already being subjected to the low-resolution typing approach. The DNA samples were subjected to conventional endpoint PCR, gel purified, cloned and sent for automated bidirectional sequencing (Eurofins Genomics, Ebersberg, Germany).

2.3.1 DNA extraction

The DNA was extracted from whole blood (for details see 2.2.1). The previously extracted DNA from Ellegaard Göttingen Minipigs #8 and #9 belonging to Cohort Sanofi 2016 were used for the examination with high resolution typing.

2.3.2 PCR amplification

High-resolution analysis of already low-resolution determined SLA genes or detection of potential novel SLA-haplotypes could be realized by PCR amplification of genomic DNA with gene locus-specific primers, followed by cloning and sequencing. Therefore, the previously extracted DNA could be applied in this SBT approach. High-resolution analysis of SLA-DRB1 and SLA-DQB1 in pigs #8 and #9 from Cohort Sanofi 2016 requested a set of particular primers (Tab. 5). To determine SLA-DRB1, a DRB1/3+ forward primer and DRB1/3- reverse primer, with sequences 5'-GCC TTC AGC CTT TTC AGG AGC C-3‘ and 5‘-ACA CAC ACT CTG CCC CCC G-3‘ (*Luetkemeier et al. 2009*), respectively, were used in the the PCR reaction. The obtained PCR product size resulted in 373 bp long DNA products. The primer-mix used to

analyse SLA-DQB1 included a DQB1/3+ forward primer: 5‘-CGG GCG GAG GCC TGA CTG-3‘ and a DQB1/3- reverse primer: 5‘-CGG CGG GCA AGC ACT CAC-3‘ (*Luetkemeier et al. 2009*), resulting in the PCR product size lenght of 400 bp.

Tab. 5: Primer set used for amplification of the SLA-DRB1 and SLA-DQB1.

Primer set	Primer	Range (bp)	Primer sequence (5' → 3')
SLA-DRB1	DRB1/3+ fw	373	GCCTTCAGCCTTTTCAGGAGCC
	DRB1/3- rev		ACACACACTCTGCCCCCCG
SLA-DQB1	DQB1/3+ fw	400	CGGGCGGAGGCCTGACTG
	DQB1/3- rev		CGGCGGGCAAGCACTCAC

fw = forward primer; rev = reverse primer; Reference: *Luetkemeier et al. 2009*.

To set up the PCR reaction, 1 µl of 50x SLA-DRB1 or SLA-DQB1 primer-mix (0.5 µl forward and reverse primer, each at 10 pmol/µl) was pipetted to the PCR reaction. The PCR reaction also contained 12.5 µl All Taq Master Mix (4x) (Qiagen) and 34.5 µl Nuclease-free H_2O. Finally, 2 µl DNA from both pigs (#8 and #9, Cohort Sanofi 2016) were added to the reaction, resulting in the total reaction volume of 50 µl. After placing the samples in the thermal cycler (T Gradient thermal cycler; Biometra), the cycling conditions needed to be adjusted due to differing annealing temperatures of the used primers. To allow the primers to anneal to the complementary DNA template, the annealing temperature was set to 68°C and 64°C for DRB1 (primer pair DRB1/3+ and DRB1/3-) and DQB1 (primer pair DQB1/3+ and DQB1/3-), respectively (Tab. 6 and 7). The PCR amplification started with initial incubation at 95°C for 5 minutes, followed by 35 cycles of 30 seconds long heating step at 94°C, 30 seconds at the appropriate annealing temperature, and 30 seconds of DNA synthesis in the elongation step at 72°C. After 35 cycles, the reactions were incubated 5 minutes at 72°C for a final extension.

Tab. 6: DRB1 thermal cycling conditions.

Step		Temperature	Duration
Initial incubation		95°C	5 minutes
35 cycles	Melting	94°C	30 seconds
	Annealing	68°C	30 seconds
	Extension	72°C	30 seconds
Hold		72°C	5 minutes

Tab. 7: DQB1 thermal cycling conditions.

Step		Temperature	Duration
Initial incubation		95°C	5 minutes
35 cycles	Melting	94°C	30 seconds
	Annealing	64°C	30 seconds
	Extension	72°C	30 seconds
Hold		72°C	5 minutes

The PCR products were analyzed by agarose gel electrophoresis and subsequently subjected to molecular cloning.

2.3.3 Agarose gel electrophoresis

For analyzing the PCR products by gel electrophoresis, a 1.5% agarose gel was used. To obtain a 1.5% agarose gel, 1.8 g of electrophoresis-grade agarose (LE Agarose, Biozym Scientific) was added to 120 ml of 1x TAE electrophoresis buffer. Subsequent gel casting steps were performed as described in section 2.2.4. with the exception of running a DNA ladder marker (FastRuler® Low Range DNA ladder, Thermo Fisher Scientific) alongside with the samples.

2.3.4 Recovery of PCR products

The QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was used to recover the PCR products from agarose gel after gel electrophoresis.

After separation of the PCR products by gel electrophoresis, the bands of interest were excised from the gel with as little of surrounding agarose as possible and transferred to a 2 ml microcentrifuge tube. Purification of the DNA from the gel material was accomplished by using the QIAquick Gel Extraction Kit (Qiagen) that utilizes a silica-membrane-based purification of DNA fragments from agarose gels (up to 400 mg gel slice) or enzymatic reactions. After adding 3 volumes of Buffer QG to 1 volume of gel. Next, the mixture was incubated for 10 minutes (or until the gel slice has completely dissolved) at 50° on a heating block combined with vortexing of the sample every few minutes to facilitate dissolving of the gel. Since the adsorption of DNA is efficient only at the correct pH, the dissolved sample needed to be controlled before the next step. The accurate pH could be confirmed based upon the color of the sample. A yellow colored sample determined by an incorporated pH indicator in Buffer QG implied an optimal pH, a

higher pH changed the color of the solution to orange or violet. For correcting the pH, 10 µl 3 M sodium acetate could be added to the sample so that the solution turned yellow again. Next, isopropanol in amount of one gel volume was added and the sample thoroughly mixed. The whole sample was pipetted onto a QIAquick spin column placed in a 2 ml collection tube and centrifuged (Biofuge Pico, Heraeus, Hanau, Germany) for 1 minute to bind the DNA to the silica membrane in the column. Due to the selective binding capacity of the silica membrane, the contaminants passed through the pores of the membrane while the DNA was bound by the silica. The DNA adsorption was only possible with concentrated chaotropic salts, which have the ability to alter the structure of water. Buffers provided by the kit have adjusted concentrations of salt needed for this procedure. The following washing step involved the addition of 750 µl of Buffer PE and a centrifugation step for 1 minute at 13,300 rpm (Biofuge Pico, Heraeus). In order to remove residual ethanol from Buffer PE entirely, the flow-through needed to be discarded, followed by centrifugation for 1 additional minute at 13,300 rpm (Biofuge Pico, Heraeus). The column with bound DNA could then be placed into a clean 1.5 ml microcentrifuge tube to get prepared for DNA elution. Therefore, 50 µl of Buffer EB were directly added to the QIAquick silica membrane and centrifuged for 1 minute at 13,300 rpm (Biofuge Pico, Heraeus). Finally, a re-elution step (as described in section 2.1.1) was an effective way to increase the amount of gel purified PCR products. The effectiveness of the DNA elution was limited by the pH with the utmost efficiency at a pH between 7.0 and 8.5. In this case, the Buffer EB ensured the required pH since its pH-value was 8.5.

2.3.5 Ligation and transformation

Molecular cloning was applied to produce a recombinant, extrachromosomal circular DNA molecule that could autonomously replicate within a microbial host. By DNA ligation, a gene of interest was physically linked to a DNA vector with blunt or cohesive ends. In this process, covalent bonds and a closed circular molecule capable of transforming a bacterial strain were formed. The recombinant plasmid in the host was then available for amplification prior to downstream application such as DNA sequencing. For the ligation reaction two different cloning systems were used: pGEM T Easy T/A cloning system (Promega Corporation) and GeneJET PCR cloning Kit (Thermo Fisher Scientific).

The 3015 bp long pGEM-T Easy Vector was used for ligation reaction of SLA-DQB genes of Ellegaard Göttingen Minipigs #8 and #9 belonging to Cohort Sanofi 2016. Sticky-End protocol for cloning PCR products with 3'-dA overhangs generated by using Taq DNA polymerase was applied (SOP at the Institute of Immunology, Vetmeduni Vienna). A ligation reaction with a total

volume of 10 µl was set up by mixing 5 µl of 2x Rapid Ligation buffer (T4 DNA Ligase), 1 µl pGEM-T Easy Vector (50 ng) and 3 µl of the purified PCR product. Finally, 1 µl T4 DNA Ligase (3 U/µl) was added to the ligation reaction, followed by incubation of 1 hour at RT.

The GeneJET PCR cloning Kit (Thermo Fisher Scientific) with a vector size of 2974 bp was used for the ligation reaction of animals #8 and #9 Cohort Sanofi 2016 for cloning the SLA-DRB genes. Following the Sticky-End protocol (SOP at the Institute of Immunology, Vetmeduni Vienna), a blunting reaction was set up using 10 µl of 2x Rapid Ligation buffer (T4 DNA Ligase), and adding 3 µl purified PCR product, together with 4 µl nuclease-free water and 1 µl DNA blunting enzyme. The mixture was incubated for 5 minutes at 70°C in a thermocycler and then cooled down for several minutes on ice. Next, 1 µl pJET1.2/blunt Cloning Vector (50 ng) and 1 µl T4 DNA Ligase (5 U/µl) were added and the ligation reaction was incubated for 30 minutes at RT.

Transformation began with introducing 2 µl pGEM-T Easy or 4 µl pJET1.2/blunt ligation reaction to 50 µl competent *Escherichia coli* (*E. coli*) cells (JM109, Promega) and mixing the cells gently by only flicking the tube. Next, the transformation mixture was incubated on ice for 20 minutes, followed by a 1-minute incubation at 42°C in a water bath. Then the transformation mixture was transfered back on ice for 2 minutes. Subsequently, 500 µl SOC medium was added to the transformation mixture and the cells were incubated for 1 hour at 37°C on a heating block (Eppendorf) with shaking at 700 rpm. For the pGEM-T Easy T/A cloning, LB/Amp^{100} plates were suplemented with 40 µl IPTG (Isopropyl β-D-1-thiogalactopyranoside) and 40 µl X-Gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galacto-pyranoside) by spreading the mixed reagents over the surface of the plates. The absorbtion of IPTG/X-Gal for 30 min at RT prior to spreading the transformation mixture allows a blue/white screening due to the presence of the LacZ gene on the pGEM-T Easy T/A cloning vector.

Whereas for the GeneJET1.2 PCR cloning the IPTG/X-Gal pre-treated LB/Amp (100 mg/ml sterile H_2O = Amp^{100}) plates were not necessary due to the lack of the LacZ gene in the pJET1.2/blunt cloning vector. With the GeneJET PCR cloning system, the positive selection of recombinant plasmids worked via the lethal gene eco47IR on the pJET1.2/blunt cloning vector. Cells that have been incubated with the SOC medium for at least one hour were subsequently plated in 2x 250 µl portions onto two LB/Amp^{100} plates and incubated overnight at 37°C.

2.3.6 Colony PCR

As two different cloning systems were used in the ligation reaction, the developmental result of bacterial colonies deriving from both vectors were quite distinguishable. Plates with colonies

containing the pGEM-T Easy vector (Promega Corporation) growed two types of bacteria. White colonies were formed by recombinant – potentially positive – clones and were subsequently analysed; whereas blue colonies did not carry the purified PCR product in the cloning vector. Using the GeneJET1.2/blunt cloning system (Thermo Fisher Scientific) allowed only the growth of recombinant clones, therefore any colony appearing on the plate could be considered as being positive and was therefore adequate for further experiments.

From colonated LB/Amp^{100} agar plates, a minimum of 6 bacterial colonies were picked with a sterile toothpick and dissolved in 100 µl nuclease-free water supplemented with Amp^{100} in 0.5 ml centrifuge tubes. To disperse the bacterial cells, the samples were placed on a shaker for 5 minutes. Next, the toothpicks were removed and discarded. For the colony PCR of selected clones, 6.25 µl of 2x TopTaq Master Mix (Qiagen) together with 0.25 µl of relevant forward primer (10pmol/µl) and 0.25 µl of relevant reverse primer (10pmol/µl) were pipetted into 0.2 ml PCR tubes. After adding 1.25 µl 10x Coral Load dye (Qiagen), 5 µl of dispensed bacteria were transferred to the PCR tube. The PCR reactions were incubated on a thermocycler and PCR was run with specific cycling conditions for SLA-DRB1 and SLA-DQB1 (Tab. 8 and 9).

Tab. 8: Colony PCR cycling conditions for SLA-DRB1.

Cycle step	Temperature	Duration	no. of cycles
1	95°C	5 minutes	
2	95°C	20 seconds	30
3	68°C	20 seconds	
4	72°C	20 seconds	
5	72°C	3 minutes	
6	15°C	hold	

Tab. 9: Colony PCR cycling conditions for SLA-DQB1.

Cycle step	Temperature	Duration	no. of cycles
1	95°C	5 minutes	
2	95°C	20 seconds	30
3	64°C	20 seconds	
4	72°C	20 seconds	
5	72°C	3 minutes	
6	15°C	hold	

For analyzing the PCR products by gel electrophoresis, a 1.5% agarose gel was used. To obtain a 1.5% agarose gel, 1.8 g of electrophoresis-grade agarose (LE Agarose, Biozym Scientific) was added to 120 ml of 1x TAE electrophoresis buffer. Subsequent gel casting steps were performed as described in section 2.2.4. with the exception of running a DNA ladder marker (FastRuler® Low Range DNA ladder, Thermo Fisher Scientific) alongside with the samples.

2.3.7 Plasmid Miniprep and restriction digest

Cell suspension of positively identified clones were inoculated in 3 ml LB/Amp^{100} liquid medium and incubated overnight at 37°C with shaking at 250 rpm. On the following day, inoculated overnight cultures were pelleted with a centrifugation step of 10 minutes at 4000 rpm (Multifuge 1 S-R, Heraeus). After discarding the remaining medium, the bacterial pellets were subjected to Plasmid-Miniprep using the ZR Plasmid Miniprep Kit Classic (D4016, Zymo Research, Irvine, CA, USA). The bacteria were resuspended in 200 µl P1 Buffer, followed by lysis of bacteria by adding 200 µl P2 Buffer. For neutralization, 400 µl P3 Buffer were added to the cleared lysate. The solution was transferred to 2 ml microcentrifuge tubes and centrifuged for 5 minutes at 13,300 rpm (Biofuge Pico, Heraeus) to get rid of needless cellular residues. The supernatant was then transferred to a Zymo-Spin IIN column being placed in a collection tube. Next, the column was washed by adding 200 µl Endo-Wash Buffer and 400 µl Plasmid Wash Buffer. After addition of each wash buffer, the Zymo-Spin IIN column was centrifuged for 1 minute at 13,300 rpm (Biofuge Pico, Heraeus) and always discarding the flow through after each washing step. Afterwards, the Zymo-Spin IIN column was dried by centrifugation for 3 minutes 13,300 rpm (Biofuge Pico, Heraeus). The column with bound Plasmid DNA was placed into a clean 1.5 ml microcentrifuge tube and 60 µl Elution Buffer EB were pipetted directly onto the matrix and centrifuged at 10,000 rpm for 3 minutes (Biofuge Pico, Heraeus). Finally, a re-elution step (as described in section 2.1.1) was an effective way to increase the amount of purified Plasmid DNA.

For restriction digest, a digestion reaction with a total volume of 10 µl was set up using 1 µl 10x enzyme-specific digestion buffer, 1 µl 10x Coral Load dye (Qiagen), 1 µl Restriction enzyme, 5 µl nuclease-free water, and 2 µl Plasmid DNA. The digestion reactions were incubated at 37°C for a minimum of 1 hour.

Considering the previous application of two different cloning systems, two different digestion reactions had to be performed. In case of using pGEM-T Easy T/A cloning system (Promega Corporation), 1 µl 10x EcoRI Buffer and 1 µl EcoRI (10 U/µl) restriction enzyme had to be used.

For plasmid DNA obtained by using the GeneJET1.2/blunt cloning Kit (Thermo Fisher Scientific), 1 µl BgIII Buffer and 1 µl BgIII (10 U/µl) restriction enzyme were applied.
For analyzing the digestion reactions by gel electrophoresis, a 1.0% agarose gel was used. To obtain a 1.0% agarose gel, 1.0 g of electrophoresis-grade agarose (LE Agarose, Biozym Scientific) was added to 100 ml of 1x TAE electrophoresis buffer. Subsequent gel casting steps were performed as described in section 2.2.4. with the exception of running a DNA ladder marker (FastRuler® Middle Range DNA ladder, Thermo Fisher Scientific) alongside with the samples.

2.3.8 Custom DNA sequencing and sequence analysis

The purified plasmid DNA of Cohort Sanofi 2016 minipig #8 and #9 were subjected to automated bidirectional sequencing with standard sequencing primers (Eurofins Genomics). Therefore, plasmid DNA of 12 positive SLA-DRB1 clones and 12 positive SLA-DQB1 clones of each animal were selected. Of each positively selected plasmid clone, 15 µl plasmid DNA was send for sequencing with the vector-specific primers.
Since we used two different cloning systems for each examined SLA class II gene, two distinct sequencing primer pairs had to be used for the custom DNA sequencing. To all plasmids formerly cloned with GeneJET PCR cloning Kit (ThermoFisher) containing SLA-DRB1 the forward primer pJET1.2 and the reverse primer pJET1.2 must be used. Samples cloned with pGEM-T Easy T/A cloning system (Promega Corporation) including SLA-DQB1 required the sequencing primers M13 uni (-21) and M13 rev (-29).
Obtained sequence data were received in Fasta format and manually processed with the BioEdit sequence alignment editor (Tom Hall, Ibis Bioscience, Carlsbad, CA, USA). To determine the exact allelic sequence of the SLA-DRB1 and SLA-DQB1 genes, the obtained sequences were blasted against sequences of the IPD-MHC Release 3.3.0.0 (2019-06-13, build 126) (*Maccari et al. 2017*).

3 RESULTS

3.1 Low-resolution typing

3.1.1 DNA concentration and quality of studied animals

The concentration of the DNA samples from all Göttingen Minipigs examined in this study were measured after elution with the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). The results of DNA concentration measurement are summarized in Tab. 10.

The DNA concentrations of the examined minipigs ranged from 31.55 to 271.4 ng/µl. The 260/280 and 260/230 ratios displayed in the Table 10 indicated the quality and purity of the extracted DNA. The ratio of absorbance at 260 nm and 280 nm estimated the purity of the measured DNA. Samples were considered as 'pure' with a ratio of 1.8-2. If the ratio was considerably lower, it suggested a possible presence of contaminants such as proteins or phenol. The absorbance at 260 nm and 230 nm should show a ratio of 2 or more to be considered as 'pure'. DNA samples. Significantly lower ratios indicated the presence of co-purified contaminants. The 260/280 ratios of the examined animals ranged from 1.69 to 2.29 with the average value of 2.01. The 260/230 ratios varied between 1.47 and 2.5 with a mean value of 2.16. Based on the information on the DNA quality it was possible to predict to what extent the resolution of the samples on the agarose gels could also be influenced by unknown contaminants or low quality of reagents.

Tab. 10: DNA concentrations and quality-indicating ratios in 69 Göttingen Minipigs.

Cohort	internal ID	DNA (ng/µl)	260/280	260/230	Cohort	internal ID	DNA (ng/µl)	260/280	260/230
Merck 2016	F01	62.4	1.96	1.69	Cohort 39	M03	75.9	1.97	2.03
	F02	83.60	1.69	2.4		M04	46.5	2.06	2.26
	F03	88.05	1.93	1.7		M05	38.6	2.02	1.93
	M01	92.40	1.94	1.81		M06	56.8	2.00	2.07
	M02	96.9	1.93	2.5		M07	31.65	2.03	1.92
Sanofi 2016	#01	53.00	1.96	1.83		M08	55.75	1.99	2.01
	#02	36.05	1.96	1.69		M09	57.85	2.01	2.03
	#03	42.75	1.91	1.47		F10	42.6	2.03	1.95
	#04	35.45	2.02	2.06		F11	67.6	2.08	2.06
	#05	31.55	1.98	2.26		F12	72.95	2.11	2.08
	#06	44.05	1.98	2.12		F13	53.65	2.10	2.05
	#07	47.5	1.96	2.24		F14	57.7	2.08	1.98
	#08	49.7	1.96	1.84		F15	69.35	2.04	1.98
	#09	42.55	1.94	1.85		F16	52.9	2.10	2.00
	#10	55.35	1.94	2.08		F17	75.3	2.09	2.07
	#11	39.75	2.00	2.11		F18	52.65	2.03	1.92
	#12	47.9	1.96	2.38	Cohort 40	F01	94.5	2.05	1.93
Cohort 38	M01	137.2	1.85	2.00		F02	76.00	2.18	2.21
	M03	92.2	1.90	2.31		F03	76.8	2.23	2.30
	M06	105.0	1.90	2.45		F04	94.7	2.23	2.27
	M07	117.6	1.88	2.39		F05	57.00	2.2	2.24
	M08	113.3	1.90	2.36		F06	88.35	2.29	2.26
	M15	127.4	1.87	2.35		F07	72.4	2.07	2.15
	M16	153.2	1.88	2.42		F08	98.5	1.91	1.85
	M19	141.4	1.90	2.33		F09	97.8	2.13	2.14
	F03	147.8	1.87	2.29		M10	65.7	2.03	2.05
	F06	107.9	1.84	2.18		M11	53.3	2.07	2.02
	F12	70.0	1.84	2.32		M12	46.6	1.85	1.87
	F13	177.4	1.88	2.31		M13	58.6	1.88	2.00
	F15	271.4	1.86	2.35		M14	85.3	2.23	2.23
	F16	115.8	1.87	2.25		M15	51.5	2.01	2.03
	F17	163.0	1.87	2.30		M16	53.75	1.95	1.94
	F18	100.4	1.86	2.38		M17	45.2	2.08	2.10
Cohort 39	M01	118.85	1.97	2.17		M18	90.65	2.06	2.07
	M02	72.65	2.00	2.08					

Male minipigs are displayed in light-green color, females in light-orange color; Minipigs with not known gender are left uncolored. The 69 Göttingen Minipigs are divided in cohorts of their origin.

3.1.2 ACTA1 Test-PCR

Before assessing the SLA class I and SLA class II gene diversity of the studied animals, a control PCR was performed to successfully target α-Actin (ACTA1) by generating PCR products with a size of 516 bp. In the PCR-SSP low-resolution typing assay we considered ACTA1 as an internal positive control. All 69 analyzed Göttingen Minipigs showed positive

amplifications for ACTA1. Representative results of ACTA1 control PCRs of Cohort 40 and Cohort Sanofi 2016 are illustrated in Figure (Fig.) 1.

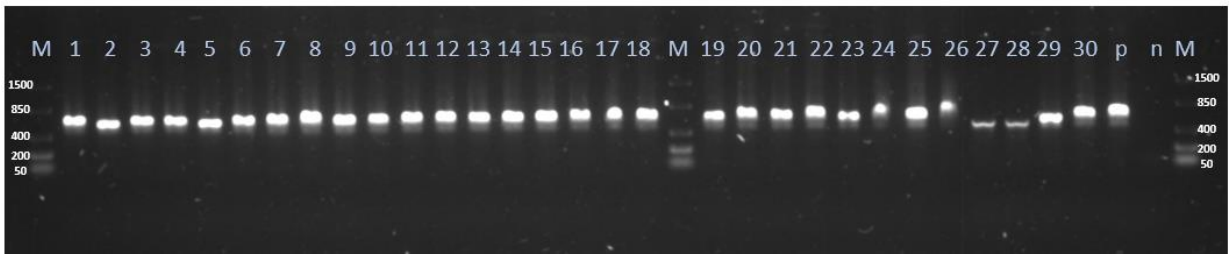


Fig. 1: ACTA1 Test-PCRs for Cohort 40 and Cohort Sanofi 2016. 4 µl of PCR products and 2 µl marker were loaded on a 3% LE agarose gel stained with GelStar™. M = Marker (FastRuler™ Low Range DNA Ladder (1500-50 bp).); 1-18 Göttingen Minipigs, Cohort 40; 19-30 Göttingen Minipigs, Cohort Sanofi 2016; p = positive control; n = no template negative control.

3.1.3 Found SLA class I genotypes and haplotypes

The obtained images from the PCR-SSP low-resolution typing approach were evaluated using the worksheets as given in 2.2.5. Results for positively identified allele groups were indicated at the corresponding coordinate on the worksheet. The list of genotypes and haplotypes for all studied animals found in this project are provided in section 7.4. An example of identified SLA class I allele groups is illustrated in Fig. 2.

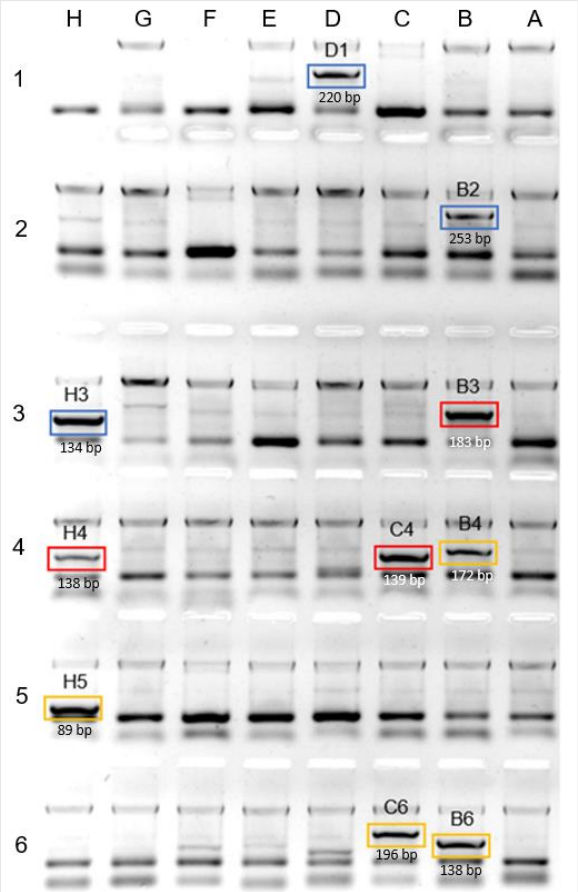


Fig. 2: Positive bands for SLA class I in low-resolution typing of Göttingen Minipig F04 belonging to Cohort 39. The entire PCR reactions were loaded on a 3% LE agarose gel stained with GelStar™. Positive SLA-1 allele groups are indicated with blue boxes, SLA-3 allele groups in red boxes and SLA-2 allele groups in yellow boxes. Positive bands are labelled with corresponding coordinates according to Table 3. For this animal, the low-resolution genotype GMP2.0 could be assigned to the haplotypes Lr-Hp 10.0 and 67.0mod. H-A = corresponding coordinates according to Tab. 3; 1-6 = corresponding coordinates according to Tab. 3; bp = base pairs.

The evaluation of each agarose gel image of all examined animals revealed nine different genotypes occurring in the five cohorts of Göttingen Miniature pigs. Assigned genotypes with corresponding allele groups and low-resolution haplotypes (Lr-Hp) for the SLA class I of the investigated animals are displayed in Tab. 11. Every inspected cohort had a minimum of four different genotypes. The Cohort 39 exhibited the maximum of six different defined genotypes.

Tab. 11: SLA class I genotypes and haplotypes found in 69 Göttingen Minipigs.

GMP	SLA-1	SLA-3	SLA-2	SLA-I Lr-Hp	Numbers
1.0	05XX/15XX	04XX	06XX + N2002	24.0mod	20
	05XX/15XX	05XX/08XX	01XX	GMP-1	
2.0	05XX	08XX	03XX	10.0	18
	15XX	05XX	01XX	67.0mod	
3.0	05XX/15XX	04XX	06XX + N2002	24.0mod	12
	08XX	05XX	01XX	49.0mod	
4.0	05XX/15XX	04XX	06XX + N2002	24.0mod	5
	05XX/15XX	08XX	01XX/03XX	GMP-2	
5.0	15XX	03XX	03XX	03.0mod	6
	08XX	05XX	blank	49.0	
7.0	05XX	08XX	03XX	10.0	4
	05XX/15XX	04XX	06XX + N2002	24.0mod	
13.0	15XX	03XX	03XX	03.0mod	2
	blank	04XX	06XX + N2002	24.0	
15.0	05XX	08XX	03XX	10.0	1
	08XX	05XX	01XX	49.0mod	
16.0	05XX	08XX	blank	10.0mod	1
	05XX/15XX	04XX	06XX + N2002	24.0mod	

GMP = Göttingen Minipig Genotype; Lr-Hp = Low resolution Haplotype; mod = modified.

Göttingen Minipig genotype GMP1.0 was the most common SLA class I genotype occurring in 20 of 69 investigated minipigs. The SLA class I genotype GMP1.0 with its corresponding Lr-Hp 24.0mod and GMP-1 was found in every inspected cohort, with the highest abundance in Cohort 39 with 8 out of 18 animals. Genotype GMP2.0 with the combination of haplotypes Lr-Hp 10.0 and 67.0mod represented the second most common genotype for SLA class I among the 69 minipigs and was found in 18 animals. Twelve minipigs were positive for the third most frequent SLA class I genotype GMP3.0, which was distributed among all cohorts except for the Cohort Merck 2016. There were also genotypes that appeared only once, namely the genotype GMP15.0 (Lr-Hp 10.0 and 49.0mod) in Cohort Sanofi 2016 and genotype GMP16.0 (Lr-Hp 10.0mod and 24.0mod) in Cohort Merck 2016. Other genotypes occurred in 2 to 6 animals (GMP4.0, GMP5.0, GMP7.0 and GMP13.0) (Tab. 11).

3.1.4 Found SLA class II genotypes and haplotypes

With the same interpretation approach, as for the SLA class I genotypes, the SLA class II genotypes were assigned. The list of genotypes and haplotypes for all studied animals found

in this project are provided in section 7.4. An example of identified alleles of SLA class II allele groups is illustrated in Fig. 3.

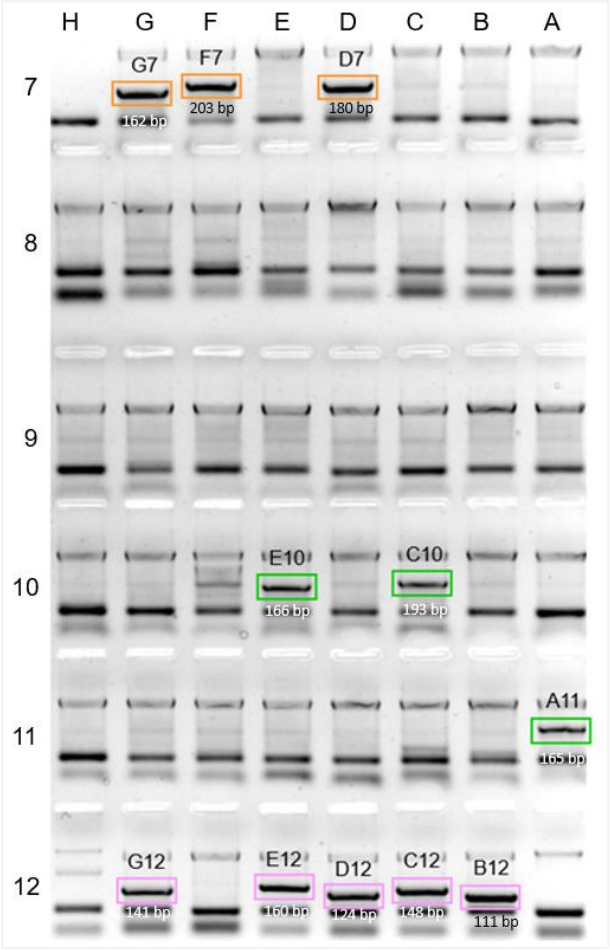


Fig. 3: Positive bands for SLA class II in low-resolution typing of Göttingen Minipig F04 belonging to Cohort 39. The entire PCR reactions were loaded on a 3% LE agarose gel stained with GelStar™. Positive SLA-DRB1 allele groups are indicated with orange boxes, SLA-DQB1 allele groups in green boxes and SLA-DQA allele groups in pink boxes. Positive bands are labelled with corresponding coordinates according to Table 4. For this animal, the low-resolution genotype GMP0.1 could be assigned to the haplotypes Lr-Hp 0.03 and 0.21. H-A = corresponding coordinates according to Tab. 4; 7-12 = corresponding coordinates according to Tab. 4; bp = base pairs.

In 69 Göttingen Minipigs we found 6 distinct SLA class II genotypes. Assigned genotypes with corresponding allele groups and low-resolution haplotypes for the SLA class II of the investigated animals are displayed in Tab. 12.

Tab. 12: SLA class II genotypes and haplotypes found in 69 Göttingen Minipigs.

GMP	DRB1	DQB1	DQA	SLA-II Lr-Hp	Numbers
0.1	01XX	05XX	04XX(+05XX)	0.21	27
	03XX	03XX (03:01)	01XX	0.3	
0.2a	01XX	05XX	04XX(+05XX)	0.21	19
	01XX	05XX	04XX(+05XX)	0.21	
0.2b	01XX	05XX	04XX(+05XX)	0.21	
	03XX	05XX	04XX	0.31mod	
0.3	01XX	05XX	04XX(+05XX)	0.21	10
	08XX	05XX	blank	0.17	
0.4	03XX(03:02)	03XX(03:02)	01XX	0.3	7
	08XX	05XX	04XX(+05XX)	0.17mod	
0.8	03XX	03XX (03:01)	01XX	0.3	5
	03XX	03XX (03:01)	01XX	0.3	
0.10	08XX	05XX	04XX(+05XX)	0.17mod	1
	08XX	05XX	04XX(+05XX)	0.17mod	

GMP = Göttingen Minipig Genotype; Lr-Hp = Low resolution Haplotype; mod = modified.

The most common genotype with the assigned number 0.1 occurred in all five minipig cohorts and was documented in 27 animals. This genotype comprises Lr-Hp 0.21 in combination with Lr-Hp 0.3. The genotype GMP0.2 was also distributed among all cohorts, with a total of 19 animals positive for this genotype, of which 18 minipigs were assigned with genotype GMP0.2a and one minipig with genotype GMP0.2b, respectively. Genotype GMP0.2a represents a homozygous Lr-Hp 0.21; whereas genotype GMP0.2b comprises Lr-Hp 0.21 combined with Lr-Hp 0.31mod. Ten out of 69 minipigs were positive for SLA class II genotype with assigned number 0.3 that carries Lr-Hp 0.21 in combination with Lr-Hp 0.17. This Lr-Hp combination was found in Cohorts 38, 39 and 40. Genotypes GMP0.4 and GMP0.8 were present in 7 and 5 minipigs, respectively. Genotype GMP0.10 is homozygous Lr-Hp 0.17mod and was present in only one animal, namely in Cohort 39.

3.1.5 Genotypes and haplotypes for all studied animals

Among 69 studied Göttingen Minipigs, 18 different genotype combinations were found. The most common genotype combination was GMP 1.0 with GMP 0.2 present in 16 of 69 Göttingen Minipigs. All SLA class I and SLA class II genotypes and haplotypes being found in the 69 studied Göttingen Minipigs are summarized in Tab. 13.

Tab.13: SLA-I and SLA-II genotypes and haplotypes of 69 Göttingen Minipigs.

Cohort	internal ID	SLA-I GMP	SLA-I Lr-Hp	SLA-II GMP	SLA-II Lr-Hp	Cohort	internal ID	SLA-I GMP	SLA-I Lr-Hp	SLA-II GMP	SLA-II Lr-Hp
Merck 2016	F01	16.0	10.0mod 24.0mod	0.2a	0.21 0.21	Cohort 39	M03	3.0	24.0mod 49.0mod	0.3	0.21 0.17
	F02	1.0	24.0mod GMP-1	0.2a	0.21 0.21		M04	1.0	24.0mod GMP-1	0.2a	0.21 0.21
	F03	13.0	03.0mod 24.0	0.8	0.3 0.3		M05	1.0	24.0mod GMP-1	0.2a	0.21 0.21
	M01	13.0	03.0mod 24.0	0.1	0.21 0.3		M06	1.0	24.0mod GMP-1	0.2a	0.21 0.21
	M02	4.0	24.0mod GMP-2	0.1	0.21 0.3		M07	4.0	24.0mod GMP-2	0.1	0.21 0.3
Sanofi 2016	#01	1.0	24.0mod GMP-1	0.1	0.21 0.3		M08	7.0	10.0 24.0mod	0.1	0.21 0.3
	#02	1.0	24.0mod GMP-1	0.1	0.21 0.3		M09	1.0	24.0mod GMP-1	0.2a	0.21 0.21
	#03	3.0	24.0mod 49.0mod	0.4	0.3 0.17mod		F10	2.0	10.0 67.0mod	0.8	0.3 0.3
	#04	2.0	10.0 67.0mod	0.1	0.21 0.3		F11	1.0	24.0mod GMP-1	0.2a	0.21 0.21
	#05	3.0	24.0mod 49.0mod	0.1	0.21 0.3		F12	5.0	03.0mod 49.0	0.10	0.17mod 0.17mod
	#06	1.0	24.0mod GMP-1	0.1	0.21 0.3		F13	2.0	10.0 67.0mod	0.1	0.21 0.3
	#07	2.0	10.0 67.0mod	0.1	0.21 0.3		F14	1.0	24.0mod GMP-1	0.1	0.21 0.3
	#08	2.0	10.0 67.0mod	0.8	0.3 0.3		F15	1.0	24.0mod GMP-1	0.2a	0.21 0.21
	#09	1.0	24.0mod GMP-1	0.2a	0.21 0.21		F16	4.0	24.0mod GMP-2	0.1	0.21 0.3
	#10	2.0	10.0 67.0mod	0.4	0.3 0.17mod		F17	5.0	03.0mod 49.0	0.4	0.3 0.17mod
	#11	2.0	10.0 67.0mod	0.1	0.21 0.3		F18	1.0	24.0mod GMP-1	0.2a	0.21 0.21
	#12	15.0	10.0 49.0mod	0.8	0.3 0.3	Cohort 40	F01	5.0	03.0mod 49.0	0.4	0.3 0.17mod
Cohort 38	M01	2.0	10.0 67.0mod	0.2a	0.21 0.21		F02	2.0	10.0 67.0mod	0.1	0.21 0.3
	M03	4.0	24.0mod GMP-2	0.1	0.21 0.3		F03	2.0	10.0 67.0mod	0.1	0.21 0.3
	M06	1.0	24.0mod GMP-1	0.2b	0.21 0.31mod		F04	2.0	10.0 67.0mod	0.1	0.21 0.3
	M07	1.0	24.0mod GMP-1	0.2a	0.21 0.21		F05	1.0	24.0mod GMP-1	0.2a	0.21 0.21
	M08	2.0	10.0 67.0mod	0.1	0.21 0.3		F06	3.0	24.0mod 49.0mod	0.3	0.21 0.17
	M15	1.0	24.0mod GMP-1	0.2a	0.21 0.21		F07	7.0	10.0 24.0mod	0.1	0.21 0.3
	M16	1.0	24.0mod GMP-1	0.2a	0.21 0.21		F08	3.0	24.0mod 49.0mod	0.3	0.21 0.17
	M19	3.0	24.0mod 49.0mod	0.3	0.21 0.17		F09	3.0	24.0mod 49.0mod	0.3	0.21 0.17
	F03	1.0	24.0mod GMP-1	0.2a	0.21 0.21		M10	3.0	24.0mod 49.0mod	0.3	0.21 0.17
	F06	2.0	10.0 67.0mod	0.8	0.3 0.3		M11	3.0	24.0mod 49.0mod	0.3	0.21 0.17
	F12	3.0	24.0mod 49.0mod	0.3	0.21 0.17		M12	2.0	10.0 67.0mod	0.1	0.21 0.3
	F13	2.0	10.0 67.0mod	0.1	0.21 0.3		M13	2.0	10.0 67.0mod	0.1	0.21 0.3
	F15	2.0	10.0 67.0mod	0.1	0.21 0.3		M14	5.0	03.0mod 49.0	0.4	0.3 0.17mod
	F16	4.0	24.0mod GMP-2	0.1	0.21 0.3		M15	1.0	24.0mod GMP-1	0.2a	0.21 0.21
	F17	3.0	24.0mod 49.0mod	0.3	0.21 0.17		M16	7.0	10.0 24.0mod	0.1	0.21 0.3
	F18	3.0	24.0mod 49.0mod	0.3	0.21 0.17		M17	7.0	10.0 24.0mod	0.2a	0.21 0.21
Cohort 39	M01	2.0	10.0 67.0mod	0.1	0.21 0.3		M18	5.0	03.0mod 49.0	0.4	0.3 0.17mod
	M02	5.0	03.0mod 49.0	0.4	0.3 0.17mod						

The 69 Göttingen Minipigs are divided in cohorts of their origin. GMP = Göttingen Minipig Genotype; Lr-Hp = Low resolution Haplotype; mod = modified.

3.1.6 Frequencies of found SLA class I and class II genotypes and haplotypes

Based on these findings, we estimated the frequencies of SLA class I and class II genotypes and haplotypes among the five cohorts of Göttingen Minipigs. The frequencies of found genotypes are listed in Tab. 14 and graphically illustrated in Fig. 4.

Tab. 14: Numerical representation of frequencies of found SLA class I and class II genotypes in 69 Göttingen Minipigs.

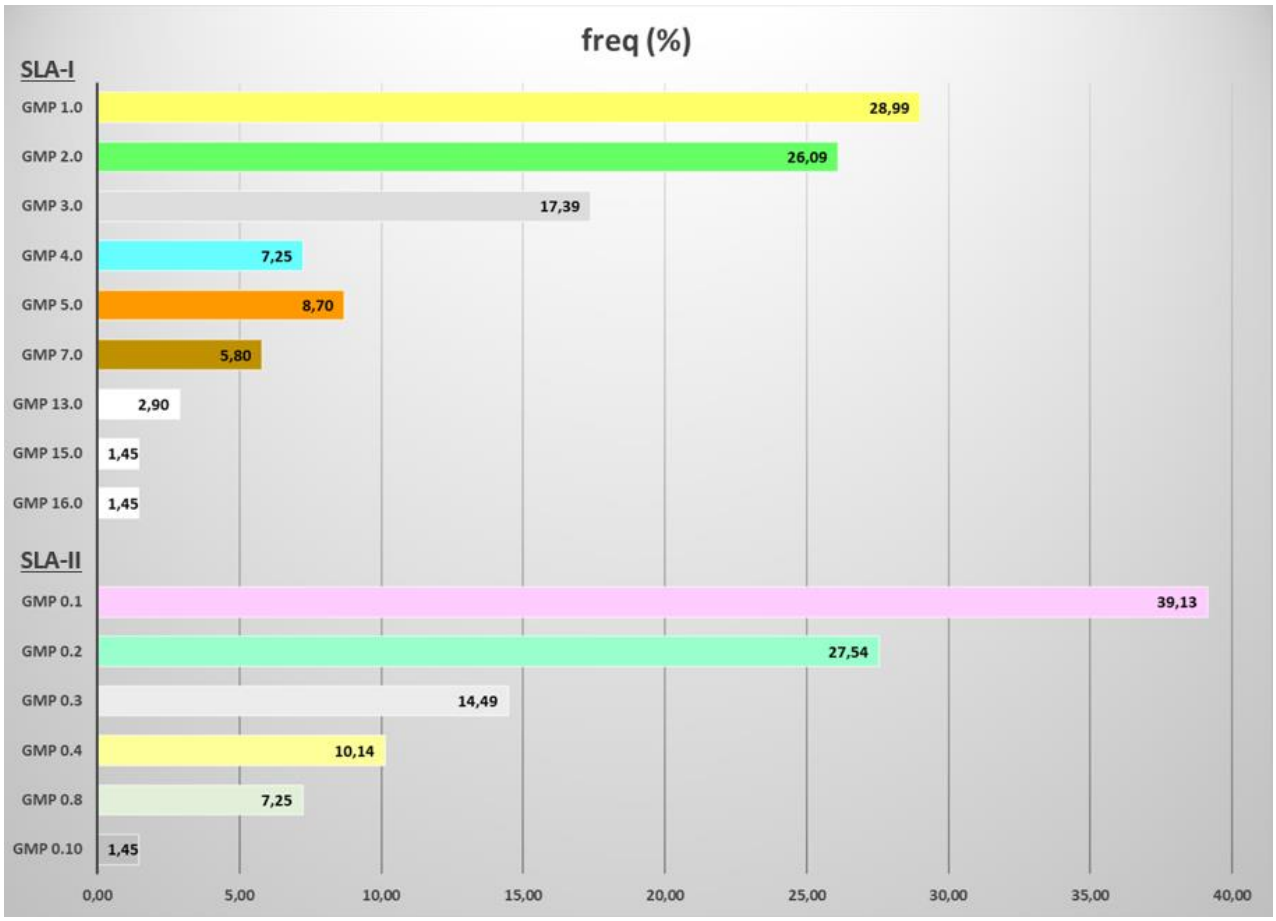
SLA-I	freq (%)	SLA-II	freq (%)
GMP1.0	28.99	GMP0.1	39.13
GMP2.0	26.09	GMP0.2	27.54
GMP3.0	17.39	GMP0.3	14.49
GMP4.0	7.25	GMP0.4	10.14
GMP5.0	8.70	GMP0.8	7.25
GMP7.0	5.80	GMP0.10	1.45
GMP13.0	2.90		
GMP15.0	1.45		
GMP16.0	1.45		

GMP = Göttingen Minipig genotype; freq = frequency (in %)

The frequency of SLA class I and class II genotypes can be visualized in the bar chart as shown in Fig. 4. The most frequent SLA class I GMP1.0 was found in 28.99% of all analyzed minipigs, followed by GMP2.0 present in 26.09% of the animals. The GMP 3.0 was found in 17.39% of the miniature pigs. Genotypes GMP15.0 and GMP16.0 occurred at the lowest frequency of 1.45%.

For SLA class II, the highest frequency of an assigned genotype was found in 39.13% of the studied pigs proving more than one-third of the tested animals positive for genotype GMP0.1. It can be speculated that this genotype may be the most frequent genotype in Göttingen Minipigs also outside of the scope of our study. The second most abundant genotype for SLA class II was found in 27.54% of the tested minipigs. Genotype GMP0.3 was found in 14.49% of the analyzed minipigs; whereas genotypes GMP0.4, GMP0.8 and GMP0.10 occurred in frequencies of 10.14%, 7.25% and 1.45%, respectively.

Fig. 4: Graphical representation of frequencies of found SLA class I and class II genotypes in 69 Göttingen Minipigs.



GMP = Göttingen Minipig genotype; freq = frequency (in %).

In the studied cohort, the nine SLA class I and six SLA class II genotypes could be assigned to ten SLA class I and five SLA class II low-resolution haplotypes (Tab. 15). The percentage of frequencies of found haplotypes in analyzed minipigs are listed in Tab. 15 and illustrated in Fig. 5.

Table 15: Numerical representation of frequencies of found SLA class I and class II haplotypes in 69 Göttingen Minipigs.

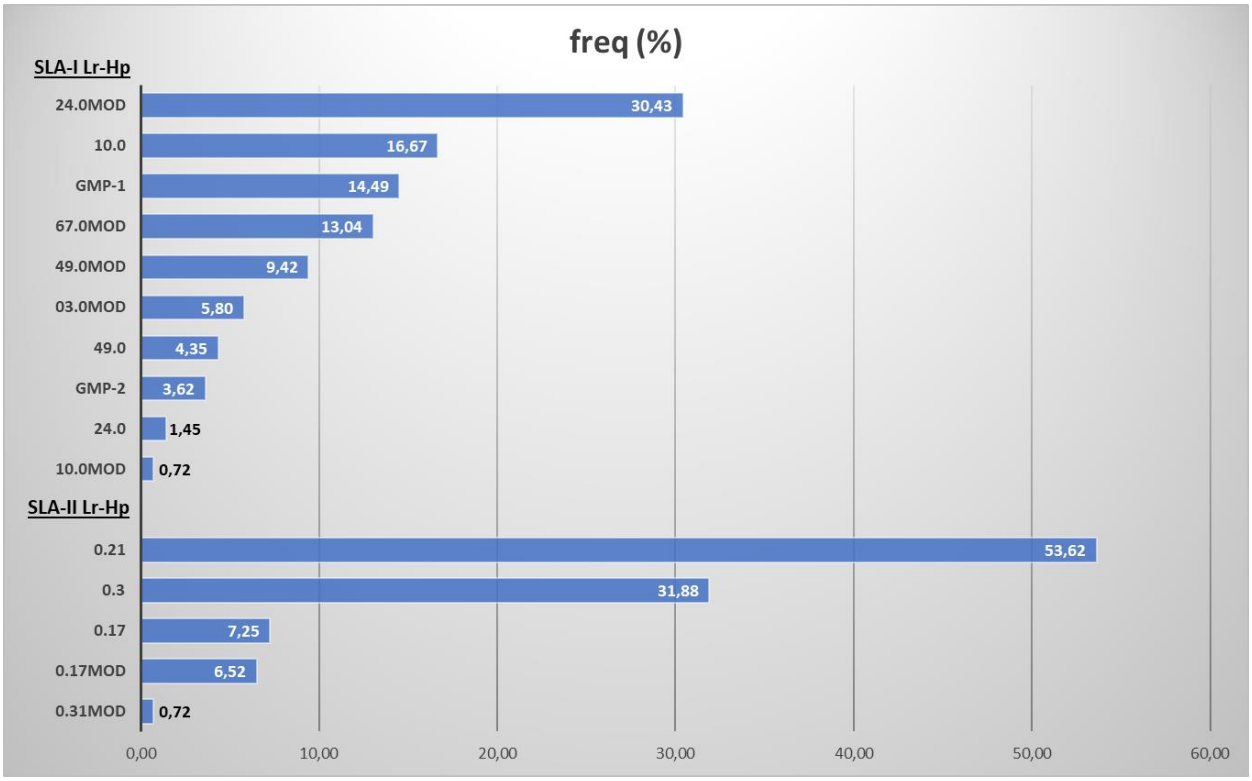
SLA-I Lr-Hp	freq (%)	SLA-II Lr-Hp	freq (%)
03.0mod	5.80	0.17	7.25
10.0	16.67	0.17mod	6.52
10.0mod	0.72	0.21	53.62
24.0	1.45	0.3	31.88
24.0mod	30.43	0.31mod	0.72
49.0	4.35		
49.0mod	9.42		
67.0mod	13.04		
GMP-1	14.49		
GMP-2	3.62		

Lr-Hp = Low-resolution Haplotype; freq = frequency (in %)

The frequency of SLA class I and class II haplotypes can be visualized in the bar chart as shown in Fig. 5. The most frequent low-resolution haplotype in SLA class I was Lr-Hp 24.mod occurring at 30.43% (Lr-Hp 24.0: 1.45%). The frequency of the second most frequent haplotype was Lr-Hp 10.0 with a frequency of 16.67% (Lr-Hp 10.0mod: 0.72%). Lr-Hp GMP-1 and Lr-Hp 67.0mod occurred at 14.49% and 13.04%, respectively. The frequencies of the haplotypes Lr-Hp 49.0mod and Lr-Hp 49.0 were 9.42% and 4.35%. Lr-Hp 3.0mod and GMP-2 occurred at 5.8% and 3.62%, respectively.

For the found SLA class II haplotypes, the frequencies of the haplotypes Lr-Hp 0.17mod and Lr-Hp 0.17 were 6.53% and 7.25%, respectively. As with Lr-Hp 10.0mod, the haplotype Lr-Hp 0.31mod was found in only 0.72% of the studied animals. The second most frequent SLA class II haplotype was Lr-Hp 0.3 with 31.88%, differing from the most frequent SLA class II haplotype by over 21%. The SLA class II haplotype Lr-Hp 0.21 was found in 53.62% of all SLA class II haplotypes and occurred with the highest frequency in all found SLA class I and II haplotypes.

Fig. 5: Graphical representation of frequencies of found SLA class I and class II haplotypes in 69 Göttingen Minipigs.



Lr-Hp = Low-resolution Haplotype; freq = frequency (in %)

3.2 High-resolution typing

Low-resolution typing provided a convenient approach for determining the SLA class I and class II genotypes and haplotypes among the studied animals. Despite this useful method, two minipigs were carrying an unclear number of different alleles and we were not able to clearly differentiate between heterozygosity and homozygosity in these animals. Based on the low-resolution typing results, they most likely exhibited a single allele-type in the SLA class II gens SLA-DRB1 and SLA-DQB1. However, as we could not exclude heterozygosity for these two animals, we decided to analyze SLA-DRB1 and SLA-DQB1 by high resolution typing. Therefore, the genomic DNA of the two Göttingen minipigs #8 and #9 belonging to Cohort Sanofi 2016 was subjected to sequence-based typing (SBT).

3.2.1 DNA concentration and quality of studied animals

For high-resolution typing of the animals #8 and #9, we used the DNA obtained for the previous analysis with the low-resolution typing method. The DNA concentration of minipigs #8 and #9

are presented in Tab. 16, including the parameters determining the quality of the extracted DNA. The measures were obtained using the Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific). In both DNA samples, the 260/280 ratios of 1.96 and 1.94 indicated high purity DNA by means of no contamination with e.g. proteins. In terms of the 260/230 ratios of 1.84 and 1.85, the outcome of the analysis indicated a minimal contamination with co-purified substances as the values were slightly below 2.

Tab. 16: DNA concentrations and quality-signifying ratios of two Göttingen Minipigs.

Cohort	internal Number	[DNA] in ng/µl	260/280	260/230
Sanofi 2016	#08	49.7	1.96	1.84
	#09	42.55	1.94	1.85

3.2.2 PCR and Cloning

PCR amplification of selected sequences, using the forward and reverse primers for SLA-DRB1 and SLA-DQB1 (see section 2.3.2, Tab. 5), were considered as successful after analysis of PCR products by agarose gel electrophoresis (Fig. 6.).

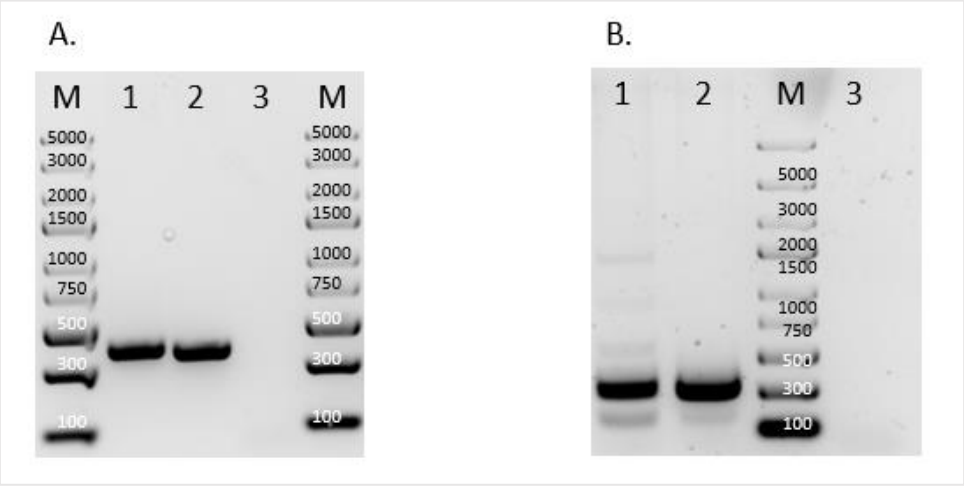


Fig. 6: PCR amplification products using forward and reverse primers for SLA-DRB1 and SLA-DQB1. 15 µl of PCR products and 4 µl marker were loaded on a 1.5% LE agarose gel stained with GelStar™. A: PCR amplification products of SLA-DRB1 using DRB1/3+ forward primer and DRB1/3- reverse primer; B: PCR amplification products of SLA-DQB1 using DQB1/3+ forward primer and DQB1/3- reverse primer. M = GeneRuler™ Express, DNA Ladder (5000-100 kb); 1: Göttingen Minipig #8 (Cohort Sanofi 2016); 2: Göttingen Minipig #9 (Cohort Sanofi 2016); 3: no template control.

Size-fractioned PCR products were gel purified and subjected to cloning. Selected bacterial colonies of successfully cloned alleles using pJET1.2/blunt Vector for SLA-DRB1 and pGEM-T Easy Vector for SLA-DQB1 were analyzed by Colony PCR (Fig. 7). When cloning the SLA-DRB1 genes, we identified 18 positive clones for both animals. Cloning of SLA-DQB1 produced 22 positive clones for minipig #8 and 24 positive clones for minipig #9. We selected 12 clones from each analyzed SLA class II genes for each minipig for subsequent plasmid miniprep and restriction digest.

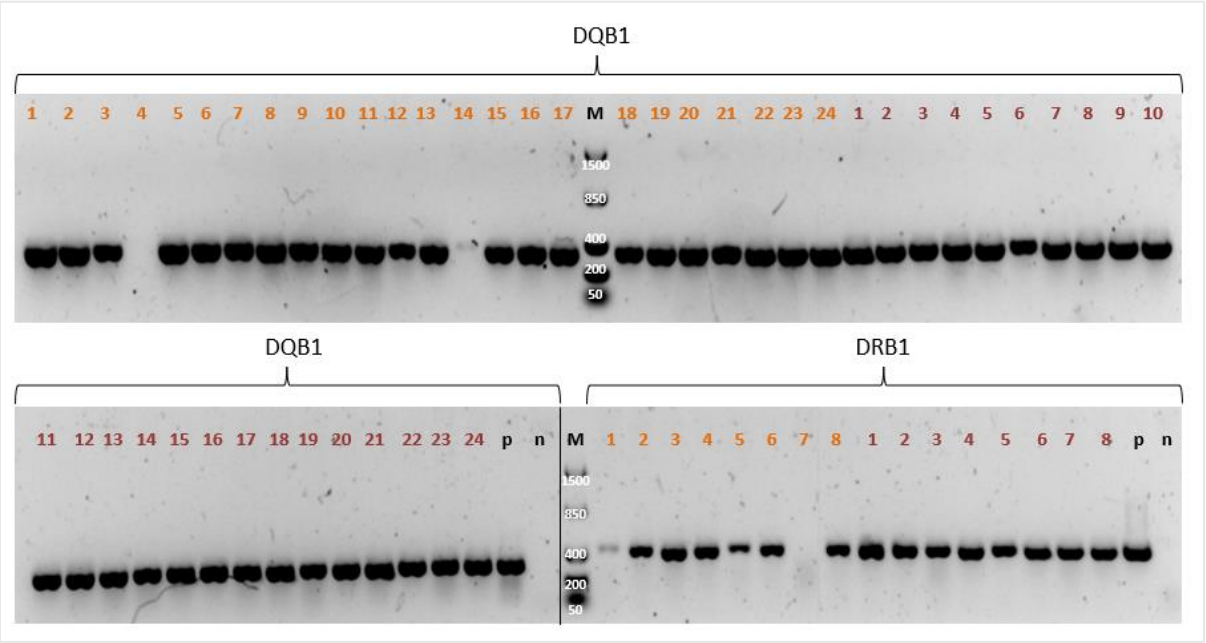


Fig. 7: Colony PCR product gel electrophoresis for SLA-DQB1 and SLA-DRB1 of Göttingen Minipigs #8 and #9 belonging to Cohort Sanofi 2016. 4 µl of colony PCR products and 2 µl marker were loaded on a 1.5% LE agarose gel stained with GelStar™. Colony PCR products of Göttingen Minipigs #8 and #9 are marked with orange and red color, respectively. M: FastRuler™ Low Range DNA Ladder (1500-50 kb); p = positive control; n = no template control.

After colony PCR, we selected 12 positive clones from each of the two minipigs for both SLA class II genes. The cell lysates of bacterial clones were inoculated in liquid overnight cultures. After plasmid miniprep, the selected clones were analyzed for correct insert size by restriction digest (Fig. 8).

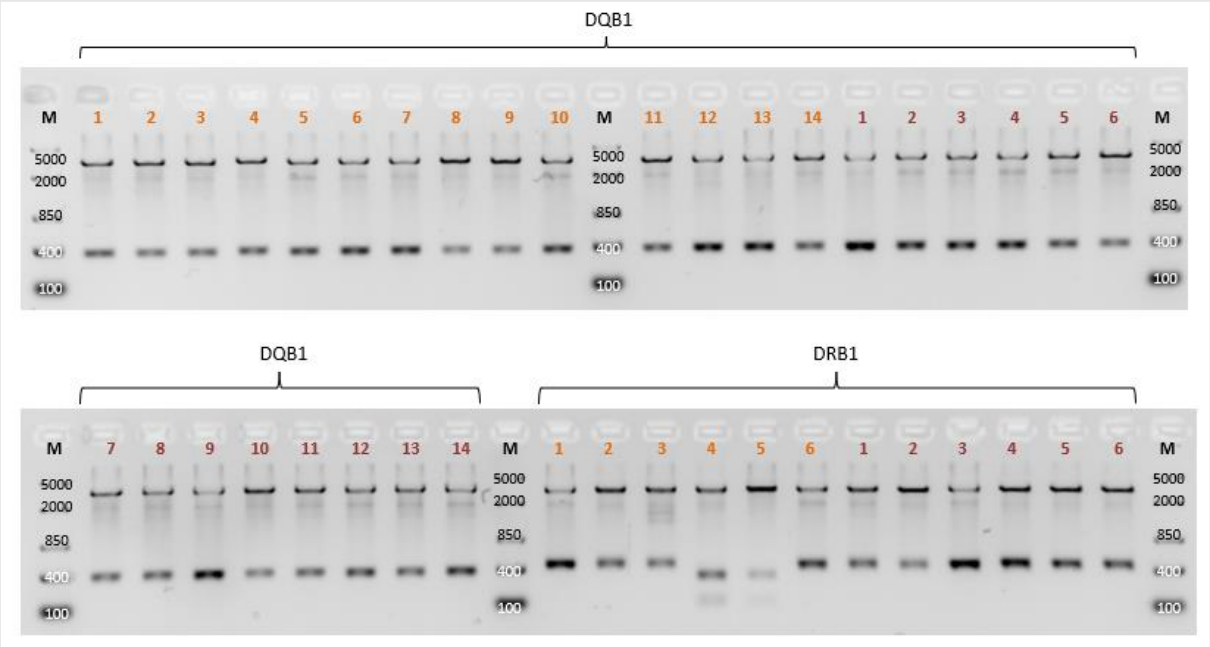


Fig. 8: Restriction digest gel electrophoresis for SLA-DQB1 and SLA-DRB1 of Göttingen Minipigs #8 and #9 belonging to Cohort Sanofi 2016. 10 µl of restriction digest reactions and 4 µl marker were loaded on a 1% LE agarose gel stained with GelStar™. Colony PCR products of Göttingen Minipigs #8 and #9 are marked with orange and red color, respectively. M: FastRuler™ Middle Range DNA Ladder (5000-100 kb).

3.2.3 SLA class II sequence analysis

Twelve SLA-DRB1 and SLA-DQB1 clones were selected after restriction digest from each animal for sequencing with standard sequencing primers and analyzed with the BioEdit software. For each clone, the vector sequence was removed from the inserts and the remaining sequences were blasted against sequences of the IPD-MHC Release 3.3.0.0 (2019-06-13, build 126).

For minipig #8 (Cohort Sanofi 2016) BLAST search results identified SLA-DRB1*03:01 and SLA-DQB1*03:01 alleles (Tab. 17). In minipig #9 (Cohort Sanofi 2016) the clones revealed the presence of SLA-DRB1*01:01 and SLA-DQB1*05:01 alleles (Tab. 17). In both animals, the identified alleles for SLA-DRB1 and SLA-DQB1 appeared singularly with absence of other potential alleles, which resulted in clarification of the homozygosity of these specific genes in minipigs #8 and #9 of the Cohort Sanofi 2016.

Tab. 17: Found SLA-DRB1 and SLA-DQB1 alleles by high-resolution typing in two Göttingen Minipigs.

Cohort	internal ID	DRB1	DQB1
Sanofi 2016	#8	03:01	03:01
	#9	01:01	05:01

Taking together the low- and high-resolution typing results for these two pigs, they have SLA class II background as summarized in Tab. 18.

Tab. 18: SLA class II background of two Göttingen Minipigs.

Cohort	internal ID	DRB1	DQB1	DQA	Hp
Sanofi 2016	#8	03:01	03:01	01XX(01:02)	0.3
	#9	01:01	05:01	04XX(+05XX)	0.21

Hp = Haplotype.

4 DISCUSSION

4.1 SLA diversity in Göttingen Minipigs

For populations to confront environmental development and transformation a genetic variety is obligatory. The diversity inside a population is expressed with detection and prediction of allelic variation, level of polymorphism and heterozygosity. To our knowledge, this is the first characterization of the swine leukocyte antigen (SLA) gene diversity in Göttingen Minipigs by low-resolution typing. The PCR-SSP typing assay is a reliable and accessible method to resolve the molecular character of the porcine MHC. Using this approach, we were able to identify distinct genotypes and haplotypes in a cohort of 69 Göttingen Minipigs. In this project, 9 distinct SLA class I genotypes and 6 different SLA class II genotypes comprising 10 and 5 different low-resolution haplotypes, were discovered among the studied cohort.

The most abundant SLA class I haplotype Lr-Hp 24.mod resembled the SLA class I gene combination SLA-1*05XX/15XX, SLA-3*04XX, SLA-2*06XX and occurred at a frequency of 30.43%. This haplotype could be derived from Lr-Hp 24.0 in which the allele group for SLA-1 was determined as 'blank', meaning still unknown. In European commercial pig lines, this haplotype was found in 26 out of 518 pigs, corresponding to a frequency of 5.02% (*Hammer et al. in preparation*).

The SLA class II haplotype Lr-Hp 0.21 was found at a frequency of 53.62% and consists of DRB1*01XX, DQB1*05XX, DQA*04XX. Again, compared to a study conducted in European commercial pig lines, this haplotype occurred in 20 out of 310 animals, resembling a frequency of 6.61% (*Hammer et al. in preparation*).

Based upon a low variety of haplotypes and their similarity, and considering the genetically background of these animals, it can be speculated that present Göttingen Minipigs already may have a restricted gene pool. However, among the studied cohort of Göttingen minipigs, a couple of potential private haplotypes were found. Commercial pigs exhibit a high SLA diversity being resembled in 50 and 35 distinct haplotypes for both, SLA class I and class II, respectively (*Hammer et al. in preparation*). This assumption is supported by the restricted occurrence of the SLA class I haplotype Lr-Hp 10.0 and the SLA class II haplotypes Lr-Hp 0.3, Lr-Hp 0.17 and Lr.Hp 0.31mod exclusively in Göttingen Minipigs. The missing occurrence of these haplotypes in commercial pig breeds, lead us to the conclusion that these four haplotypes represent potential private haplotypes in Göttingen minipigs.

4.2 Confirmatory potential of the Sequence-based typing approach

As recognized during the investigation of the occuring SLA alleles and allele groups in miniature pigs being analyzed in this project, the low-resolution typing using SSP-PCR presents a convenient and relatively accessible strategy for the molecular characterization of the porcine MHC. With a number of different primer combinations it allows an examination of the presence of various alleles and allele groups leading to a more detailed picture summarizing occurring SLA class I and class II genes. This method elucidated the SLA region of the porcine genome and enabled the recognition of appearing alleles in various pig breeds (*Ho et al. 2009, Ho et al. 2010, Essler et al. 2013, Pedersen et al. 2014, Gimsa et al. 2017, Hammer et al. in preparation*). However, this approach is highly dependent on the selection of used primers and may generate inaccurate and occasionally insufficient results. Hence, sequence-based typing (SBT) strategy offers a thorough analysis of such misinterpretation and determines the alleles that could not be specifically identified by low-resolution typing. The method is time-consuming, it includes cloning, but for the resulting accuracy the SBT approach may be an exceptionally useful tool for designating of concrete alleles, thus assigning of SLA haplotypes.

Proved by precise assessment of exact alleles present in the swine leukocyte antigen complex, the high-resolution typing approach provided a powerful way of molecular characterization of unknown alleles. The results of such typing are mostly explicit and contribute to detection and description of specific sequences. Thus, high-resolution typing presents an effective supplementary method in case of insufficient data acquired by low-resolution typing.

4.3 Implications of the SLA background of Göttingen Minipigs for its role as an animal model in biomedical research

Biomedical research is working towards unraveling the enormous impact that MHC has on the response of a human body to a received transplant. Differences in MHC between donor and recipient cause damaging of the immune responses that induce rejection of a graft after transplantation. With such incompatible MHC genes, the recipient is inflicted with acute cellular rejection leading to immediate graft loss or development of a chronic rejection that induces in most cases a late graft failure.

Thus, understanding MHC function and immunologically initiated processes could eventually evocate an absolute solution to these complications. In this matter, utilization of Göttingen Minipigs with defined genetically background that have distinguished significant similarities

with the human body and its physiological, immunological and pathological mechanisms, could be highly beneficial in transplant studies in the near future.

4.4 Conclusions and outlook

In this initial description of the SLA background of Göttingen Minipigs, we successfully applied low-resolution PCR-SSP typing assay to a cohort of 69 Göttingen Minipigs deriving from the population being housed at the company Ellegaard (Dalmose, Denmark).

Based upon a low variety of haplotypes and their similarity, considering the genetically background of these animals, the conjecture is, that the Göttingen Minipigs have a restricted gene pool. The limited number of found genotypes and haplotypes together with their frequencies and their resemblance point towards a restricted SLA diversity in this pig breed, which could be a limiting factor in later mismatch donor allo-transplant studies.

Among the studied Göttingen minipigs, in total four potential private haplotypes were found.

As a crossbreed of the Minnesota minipig and the German Landrace pig with the Vietnamese pot-bellied pig, which is not entirely characterized, there is a possibility of presence of novel haplotypes and alleles that are yet to be defined. With the high-resolution sequence-based typing approach, that allows detection and confirmation of such haplotypes and alleles, novel haplotypes and alleles can be eventually annotated based on the obtained sequence data. Such future experiments can help revising and refinement of the SLA-typing primer panel and will consequently lead to enhancement of the low-resolution PCR-SSP typing assay.

5 SUMMARIES

5.1 Summary

A cluster of highly polymorphic genes named major histocompatibility complex (MHC) plays an important role regarding immunological responses to pathogens but also to self-produced peptides circulating in the body. Porcine MHC, so called Swine Leukocyte Antigen (SLA), has two major classes of molecules that are mainly expressed on the surface of cells. The SLA class I molecules can be found on all nucleated cells and are associated with intracellular pathogens. The SLA class II molecules are expressed on the surface of specialized antigen-presenting cells and mostly present extracellular antigens. Because of their enormous influence on transplant rejection, typing of SLA rises in biomedical research. Göttingen Minipigs are for their genetically background and great similarity with human MHC very suitable animal model in allo- and xenotransplantation studies. The molecular characterization of SLA can be analyzed with low-resolution typing methods such as PCR using sequence specific primers (PCR-SSP), or sequence-based high-resolution typing strategies (SBT). In this study, 69 Göttingen Minipigs were typed using PCR-SSP, of which two minipigs were analyzed further with SBT approach examining present SLA-DRB1 and SLA-DQB1 alleles for confirmation of homozygosity of the animals.

Low-resolution typing revealed 9 SLA class-I and 6 SLA class-II genotypes, and 10 distinct SLA class-I and 5 distinct SLA class-II haplotypes among all 69 minipigs with frequencies of 30.43% and 53.62% of most common haplotypes Lr-Hp 24.0mod (SLA class-I) and Lr-Hp 0.21 (SLA class-II), respectively. High-resolution typing of minipigs #8 and #9 belonging to Cohort Sanofi 2016 identified SLA-DRB1*03:01 and SLA-DQB1*03:01 alleles in Sanofi minipig #8 and SLA-DRB1*01:01 and SLA-DQB1*05:01 alleles in Sanofi minipig #9 confirming homozygosity of these minipigs. Application of both typing methods led to affirmation of reliability of SBT approach that can be used in further examination and subsequent assignment of haplotypes of studied animals.

5.2 Zusammenfassung

Eine Gruppe von hochpolymorphen Genen genannt Haupthistokompatibilitätskomplex (Major Histocompatibility Complex, MHC) spielt eine wichtige Rolle bei immunologischen Reaktionen auf Krankheitserreger, aber auch auf selbstproduzierte, im Körper zirkulierende Peptide. Der MHC beim Schwein, das so genannte Swine Leukocyte Antigen (SLA), hat zwei Hauptklassen von Molekülen, die hauptsächlich auf der Oberfläche von Zellen exprimiert werden. Die SLA-Klasse-I-Moleküle treten auf allen kernhaltigen Zellen auf und sind mit intrazellulären Pathogenen assoziiert. Die SLA-Klasse-II-Moleküle werden auf der Oberfläche spezialisierter antigenpräsentierender Zellen exprimiert und präsentieren meist extrazelluläre Antigene. In der Transplantationsforschung steigt die Nachfrage nach der SLA-Typisierung aufgrund ihres enormen Einflusses auf die Transplantatabstoßung. Göttingen Minipigs sind aufgrund ihres genetischen Hintergrunds und der großen Ähnlichkeit des SLAs mit dem menschlichen MHC ein besonders geeignetes Tiermodell in Allo- und Xenotransplantationsstudien. Die molekulare Charakterisierung von SLAs kann mit Low-Resolution-Typisierungsmethoden wie der PCR mit sequenzspezifischen Primern (PCR-SSP) oder sequenz-basierten High-Resolution-Typisierungsstrategien (SBT) analysiert werden. In dieser Studie wurden 69 Göttinger Minipigs mit PCR-SSP typisiert, von welchen zwei Minipigs mit dem SBT-Ansatz weiter analysiert wurden, um die anwesenden SLA-DRB1- und SLA-DQB1-Allele zur Überprüfung der Homozygotie der Tiere zu untersuchen.

Die Typisierung mit dem Low-Resolution-Verfahren ergab 9 SLA-Klasse-I- und 6 SLA-Klasse-II-Genotypen, und 10 verschiedene SLA-Klasse-I und 5 verschiedene SLA-Klasse-II Haplotypen der 69 Minipigs mit einer Häufigkeit von 30.43% bzw. 53.62% der häufigsten Haplotypen Lr-Hp 24.0mod (SLA-Klasse-I) und Lr-Hp 0.21 (SLA-Klasse-II). High-Resolution Typisierung der Minipigs #8 und #9 von der Kohorte Sanofi 2016 identifizierte SLA-DRB1*03:01 und SLA-DQB1*03:01 Allele in Sanofi Minipig #8 und SLA-DRB1*01:01 und SLA-DQB1*05:01 Allele in Sanofi Minipig #9, die die Homozygotie der Minipigs bestätigen. Die Anwendung beider Typisierungsmethoden führte zu einer Bestätigung der Zuverlässigkeit der SBT-Methode, die für die weitere Analyse und anschließende Zuordnung von Haplotypen der untersuchten Tiere verwendet werden kann.

6 REFERENCES

6.1 Research papers

Ando, A., Ota, M., Sada, M., Katsuyama, Y., Goto, R., Shigenari, A., Kawata, H., Anzai, T., Iwanaga, T., Miyoshi, Y., Fujimura, N., Inoko, H. (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

Bollen, P., Ellegaard, L. (1997): The Göttingen Minipig in Pharmacology and Toxicology. Pharmacology & Toxicology, 80: 3-4

Brocke, P., Garbi, N., Momburg, F., Hämmerling, G.J. (2002): HLA-DM, HLA-DO and tapasin: functional similarities and differences. Curr. Opin. Immunol., 14: 22-9

Call, M. E., Pyrdol, J., Wiedmann, M., Wucherpfennig, K. W. (2002): The organizing principle in the formation of the T cell receptor-CD3 complex. Cell, 111: 967–979

Chardon, P., Renard, C., Vaiman, M. (1999): The major histocompatibility complex in swine. Immunological Reviews, 167: 179-192

Dong, G., Wearsch, P. A., Peaper, D. R., Cresswell, P., Reinisch, K. M. (2009): Insights into MHC class I peptide loading from the structure of the tapasin-ERp57 thiol oxidoreductase heterodimer. Immunity, 30: 21–32

Essler, S. E., Ertl, W., Deutsch, J, Ruetgen, B. C., Groiss, S., Stadler, M., Wysoudil, B., Gerner, W., Ho, C., Saalmueller, A. (2013): Molecular characterization of swine leukocyte antigen gene diversity in purebred Pietrain pigs. Anim. Genet., 44: 202-205

Figueiredo, C., Carvalho Oliveira, M., Chen-Wacker, C., Jansson, K., Höffler, K., Yuzefovych, Y., Pogozhykh, O., Jin, Z., Kühnel, M., Jonigk, D., Wiegmann, B., Sommer, W., Haverich, A., Warnecke, G., Blasczyk, R. (2019): Immunoengineering of the Vascular Endothelium to Silence MHC Expression During Normothermic Ex Vivo Lung Perfusion. Hum. Gene Ther., 30: 485-496

Fiorillo, Maria Teresa (2017): HLA Class I or Class II and Disease Association: Catch the Difference If You Can. Frontiers in immunology, 8: 1475.

Forman, J. (1984): T Cells, the MHC, and Function. Immunological Reviews, 81: 203-220

Gao, C., Quan, J., Jiang, X., Li, C., Lu, X., & Chen, H. (2017): Swine Leukocyte Antigen Diversity in Canadian Specific Pathogen-Free Yorkshire and Landrace Pigs. Frontiers in immunology, 8: 282

Garamszegi, László Zsolt. (2014): Global distribution of malaria-resistant MHC-HLA alleles: the number and frequencies of alleles and malaria risk. Malaria journal, 13: 349

Gimsa, U., Ho, C.S., Hammer, S.E. (2017): Preferred SLA class I/Class II haplotype combinations in German Landrace pigs. Immunogenetics, 69: 39-47

Hammer, S.E., Gimsa, U., Groiss, S., Stadler, M., Duckova, T., Jensen-Waern, M., Sobrino, F., Saalmüller, A., Ho, C-S. Molecular characterization of swine leukocyte antigen (SLA) gene diversity in European commercial pigs.

Hammer, S.E., Ho, C.S, Ando, A., Rogel-Gaillard, C., Charles, M., Tector, M., Tector, A.J., Lunney, J.K. 2020. Importance of the MHC (SLA) in swine health and biomedical research. Ann Rev Anim Biosci. 2020 Feb 15;8:171-198. doi: 10.1146/annurev-animal-020518-115014. Epub 2019 Dec 17.

Heckel, T., Schmucki, R., Berrera, M., Ringshandl, S., Badi, L., Steiner, G., Certa, U. (2015): Functional analysis and transcriptional output of the Göttingen minipig genome. BMC genomics, 16: 932

Hedrick, P.W. (2002): Pathogen resistance and genetic variation at MHC loci. Evolution, 56: 1902-1908

Ho, C.S., Rochelle, E.S., Martens, G.W., Schook, L.B., Smith, D.M. (2006): Characterization of swine leukocyte antigen polymorphism by sequence-based and PCR-SSP methods in Meishan pigs. Immunogenetics, 58: 873–882

Ho, C.S., Lunney, J., Franzo-Romain, M., Martens, G., Lee, Y.J., Lee, J.H., (2009): Molecular characterization of swine leucocyte antigen class I genes in outbred pig populations. Anim. Genet., 40: 468–78

Ho, C.S., Lunney, J., Lee, J.H., Franzo-Romain, M., Martens, G., Rowland, R., (2010): Molecular characterization of swine leucocyte antigen class II genes in outbred pig populations. Anim. Genet., 41: 428–32

Karnes, Jason H. (2017): Phenome-wide scanning identifies multiple diseases and disease severity phenotypes associated with HLA variants. Science translational medicine, 9: 389

Kulski, J.K., Shiina, T., Anzai, T., Kohara, S., Inoko, H. (2002): Comparative genomic analysis of the MHC: the evolution of class I duplication blocks, diversity and complexity from shark to man. Immunological Reviews, 190: 95-122

Le, M.T., Choi, H., Choi, M., Nguyen, D.T., Kim, J., Seo, H.G., Cha, S., Seo, K., Chun, T., Schook, L.B., Park, C. (2012): Comprehensive and high-resolution typing of swine leukocyte antigen DQA from genomic DNA and determination of 25 new SLA class II haplotypes. Tissue Antigens, 80: 528-535

Liepe, J., Marino, F., Sidney, J., Jeko, A., Bunting, D.E., Sette, A., Kloetzel, P.M., Stumpf, M.P., Heck, A.J., Mishto, M. (2016): A large fraction of HLA class I ligands are proteasome-generated spliced peptides. Science, 354: 354-358

Lokki, M-L, Paakkanen, R. (2019):The complexity and diversity of major histocompatibility complex challenge disease association studies. HLA, 93: 3-15

Luetkemeier, E.S., Malhi, R.S., Beever, J.E., Schook, L.B. (2009): Diversification of porcine MHC class II genes: evidence for selective advantage. Immunogenetics, 61: 119-29

Lunney, J.K., Ho C.S., Wysocki M., Smith D.M. (2009): Molecular genetics of the swine major histocompatibility complex, the SLA complex. Dev. Comp. Immunol., 33 :362–74

Maccari, G., Robinson, J., Ballingall, K., Guethlein, L.A., Grimholt, U., Kaufman, J., Ho, C.S., De Groot, N.G., Flicek, P., Bontrop, R.E., Hammond, J.A., Marsh, S.G.E. (2017): IPD-MHC 2.0: an improved inter-species database for the study of the major histocompatibility complex. Nucleic Acids Res., 45: D860–D864

Matsumura, M., Fremont, D.H., Peterson, P.A., Wilson, I.A. (1992): Emerging principles for the recognition of peptide antigens by MHC class I molecules. Science, 257: 927

Neefjes, J., Jongsma, M.L., Paul, P., Bakke, O. (2011): Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat. Rev. Immunol., 11: 823–36

Olerup, O., Aldener, A. and Fogdell, A. (1993): HLA-DQB1 and -DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. Tissue Antigens, 41: 119-134

Pedersen, L. E., Jungersen, G., Sorensen, M. R., Ho, C. S., Vadekaer, D. F. (2014): Swine Leukocyte Antigen (SLA) class I allele typing of Danish swine herds and identification of commonly occurring haplotypes using sequence specific low and high resolution primers. Veterinary immunology and immunopathology, 162: 108–116

Pos, W., Sethi, D.K., & Wucherpfennig, K.W. (2013): Mechanisms of peptide repertoire selection by HLA-DM. Trends in immunology, 34: 495–501.

Priou-Guzylack, L., Salmon, H. (2008). Membrane markers of the immune cells in swine: an update. Vet. Res., 39: 54

Renard, C., Hart, E., Sehra, H., Beasley, H., Coggill, P., Howe, K. (2006): The genomic sequence and analysis of the swine major histocompatibility complex. Genomics, 88: 96–110

Roberts, S.C. (2009): Complexity and context of MHC-correlated mating preferences in wild populations. Molecular Ecology, 18: 3121-3123

Rock, K. L., Reits, E., Neefjes, J. (2016): Present Yourself! By MHC Class I and MHC Class II Molecules. Trends in immunology, 37: 724–737

Sadegh-Nasseri, S., Chen, M., Narayan, K., Bouvier, M. (2008): The convergent roles of tapasin and HLA-DM in antigen presentation. Trends in immunology, 29: 141–147

Schook, L.B., Collares, T.V., Darfour-Oduro, K.A., De, A.K., Schlacgtschneider, K.M., Seixas, F.K. (2015): Unraveling the swine genome: implications for human health. Annual Review of Animal Biosciences; 3: 219-44

Shrestha, S. (2015): Imputation of class I and II HLA loci using high-density SNPs from ImmunoChip and their associations with Kawasaki disease in family-based study. International journal of immunogenetics, 3: 140-6

Slager, Rebecca E. (2012): Genetics of asthma susceptibility and severity. Clinics in chest medicine, 3: 431-43

Smith, D., Lunney, J. , Martens, G. , Ando, A. , Lee, J. , Ho, C. , Schook, L. , Renard, C. and Chardon, P. (2005): Nomenclature for factors of the SLA class-I system, 2004. Tissue Antigens, 65: 136-149

Sommer, S. (2005): The importance of immune gene variability (MHC) in evolutionary ecology and conservation. Frontiers in zoology, 2: 16

Sørensen, M. R., Ilsøe, M., Strube, M. L., Bishop, R., Erbs, G., Hartmann, S. B., Jungersen, G. (2017): Sequence-Based Genotyping of Expressed Swine Leukocyte Antigen Class I Alleles by Next-Generation Sequencing Reveal Novel Swine Leukocyte Antigen Class I Haplotypes and Alleles in Belgian, Danish, and Kenyan Fattening Pigs and Göttingen Minipigs. Frontiers in immunology, 8: 701

Spurgin, L.G., Richardson, D.S. (2010): How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. Proceedings. Biological sciences, 277: 979–988

Sullivan, J.A., Oettinger, H.F., Sachs, D.h., Edge, A.S. (1997): Analysis of polymorphism in porcine MHC class I genes: alternations in signals recognized by human cytotoxic lymphocytes. J. Immunol., 159: 2318-26

Tennant, L.M., Renard, C., Chardon, P., Powell, P.P. (2007): Regulation of porcine classical and nonclassical MHC class I expression. Immunogenetics, 59: 377-89

Vaiman, M., Chardon, P., Rothschild, M.F. (1998): Porcine major histocompatibility complex. Rev. Sci. Tech., 17: 95–107

Wakeman, D.R., Crain, A.M., Snyder, E.Y. (2006): Large animal models are critical for rationally advancing regenerative therapies. Regenerative medicine, 1: 405–413

Wieczorek, M., Sticht, J., Stolzenberg, S., Günther, S., Wehmeyer, C., El Habre, Z., Freund, C. (2016): MHC class II complexes sample intermediate states along the peptide exchange pathway. Nature communications, 7: 13224

Wieczorek, M., Abualrous, E. T., Sticht, J., Álvaro-Benito, M., Stolzenberg, S., Noé, F., Freund, C. (2017): Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation. Frontiers in immunology, 8: 292

Williams, A.P., Peh, C.A., Purcell, A.W., McCluskey, J., Elliot, T. (2002): Optimization of the MHC class I peptide cargo is dependent on tapasin. Immunity., 16: 509-20

Yin, Xianyong (2015): Genome-wide meta-analysis identifies multiple novel associations and ethnic heterogeneity of psoriasis susceptibility. Nature communications, 6: 6916

Zhang, N., Qi, J., Feng, S., Gao, F., Liu, J., Pan, X., Gao, G. F. (2011): Crystal structure of swine major histocompatibility complex class I SLA-1 0401 and identification of 2009 pandemic swine-origin influenza A H1N1 virus cytotoxic T lymphocyte epitope peptides. Journal of virology, 85: 11709–11724

6.2 Books

Murphy K. (2017): Janeway`s Immunobiology 9th edition, New York: Garland Science

Janeway CA Jr (2001): Immunobiology 5th edition, New York: Garland Science

7 APPENDIX

7.1 Abbreviations

A	Adenine
ACTA1	Actin alpha
Amp	Ampicillin
β2-microglobulin	beta-2 microglobulin
bp	Base pairs
C	Cytosine
CD	Cluster of Differentiation
DC	dendritic cell
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ER	Endoplasmic reticulum
E. col	Escherichia coli
Fig.	Figure
G	Guanine
gDNA	genomic DNA
GMP	Göttingen Miniature pig, Göttingen Minipig
HLA	Human leukocyte antigen
Ig	Immunoglobulin
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilo base pairs (1,000 base pairs)
lacZ	Lactose-Z
LB	Luria/Lysogeny broth
LE	Low electro-endosmosis
Lr-Hp	Low-resolution Haplotype
MHC	Major histocompatibility complex
MS	Microsatellite
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
rpm	Rounds per minute

RT	Room temperature
SBT	sequence-based typing
SLA	Swine leukocyte antigen
SOP	Standard Operating Procedure
SSP	sequence-specific primer
T	Thymidine
Tab.	Table
TAE buffer	Tris/acetate/EDTA electrophoresis buffer
TCR	T- cell receptor
UV	Ultraviolet
X-Gal	5-Brom-4-chlor-3-indoxyl-β-D-galactopyranoside

7.2 Materials and Reagents

4x All Taq Master Mix (Qiagen, Hilden, Germany)

Ampicillin^{100} (Carl Roth, Karlsruhe, Germany)

Working Solution: 100 µg/µl

Final concentration: 100 µg/ml

Biozym LE Agarose (Biozym Scientific; Hessisch Oldendorf, Germany)

CloneJET® PCR Cloning Kit (ThermoFisher Scientific, Waltham, MA, USA)

K1232 pJET1.2/blunt Cloning vector (50 ng/µl)

2x Reaction Buffer

T4 DNA Ligase (5 u/µl)

DNA Blunting Enzyme

pJET1.2. Forward Sequencing Primer (10 µM)

pJET1.2. Reverse Sequencing Primer (10 µM)

10x Coral Load dye (Qiagen)

DEPC-treated, nuclease-free water (Sigma-Aldrich Inc., Saint-Louis, MO, USA)

dNTP-mixture (Fermentas via Thermo Fisher Scientific Inc, Waltham, MA, USA), Stock solution: 10 mM each dNTP

EcoRI (10U/µl) (Thermo Fisher)

10x Buffer EcoRI: 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 100 mM NaCl, 0.02 % Triton X-100, 0.1 mg/ml BSA

EL Buffer (Qiagen)

96 % Ethanol (VWR International, Vienna, Austria)

E.Z.N.A Blood DNA Mini Kit (Omega Bio-tek, Inc., Nocross, GA, USA)

OB Protease Solution

BL Buffer

HBC Buffer

DNA Wash Buffer

Elution Buffer

FastRuler™ Low Range DNA Ladder (Fermentas)

FastRuler™ Middle Range DNA Ladder (Fermentas)

GeneRuler™ Express DNA Ladder (Thermo Fisher)

GelStar® Nucleic Acid Gel Stain (Cambrex Corporation, East Rutherford, NJ, USA), Stock solution: 10 000x Working solution: 400x

2x HotStar Taq DNA Polymerase Mix (Qiagen)

IPTG (Sigma-Aldrich)

Working concentration: 4 mM/L

JM109 Competent cells (>107 cfu/µg, Promega)

LB (Luria or Lenox broth) medium (pH 7.0)

10 g tryptone

5 g yeast extract

5 g NaCl

100 µg/ml Ampicillin

LB plates with Ampicillin (LB/Amp^{100})

15 g agar in 1 litre of LB medium

100 µg/ml Ampicillin

pGEM T®-Easy Vector System (Promega)

pGEM T®-Easy Vector (50 ng/µl)

T4-Ligase

2x Rapid Ligation Buffer, T4 DNA Ligase

Plasticware

0.1-10 µl ep.T.I.P.S® Standard/Bulk (Eppendorf AG, Hamburg, Germany)

0.5-20 µl ep.T.I.P.S® Standard/Bulk (Eppendorf AG)

10 µl LRF Filter Tips, Sterile (Golden Gate Bioscience, CA, USA)

30 µl LRF Filter Tips, Sterile (Golden Gate Bioscience)

100 µl LRF Filter Tips, Sterile (Golden Gate Bioscience)

1000 µl LRF Filter Tips, Sterile (Golden Gate Bioscience)

0.5 ml microcentrifuge tube (Greiner Group, Kremsmünster, Austria)

1.5 ml microcentrifuge tube (Greiner Group)

2.0 ml microcentrifuge tube (Greiner Group),

1.5 ml Safe-Lock Tubes (Eppendorf AG)

2.0 ml Safe-Lock Tubes (Eppendorf AG)

15 ml conical centrifuge tubes (Greiner Group)

QIAquick Gel Extraction Kit (Qiagen)

Buffer EB

Buffer PE

Buffer QG

RNAse A (Sigma-Aldrich)

SOC medium

2 % (w/v) tryptone

0.5 % (w/v) yeast extract

10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose

1x TAE (Tris/acetate/EDTA) electrophoresis buffer, pH 8.2-8.4 (at 25°C)

40 mM Tris acetate

1 mM EDTA

2x TopTaq Master Mix (Qiagen)

X-Gal (Thermo Fisher)

Working concentration: 2 mM/l

ZR Plasmid Miniprep Kit Classic (Zymo Research, Irvine, CA, USA)

P1 Buffer

P2 Buffer

P3 Buffer

Endo-Wash Buffer

Plasmid Wash Buffer

Elution Buffer EB

7.3 PCR SSP-Primers used in this study

Tray Pos Row	Tray Pos Col	Group Specificity	Allele Specificity	Product Size (bp)	Primer Set ID	Primer ID	Primer Sequence (5'→3') (Upper = forward primer; lower = reverse primer; underlined = intron sequence; lower case = intentional mismatch)	3' Pos	Minor Sequence Mismatch	Intentional Mismatch	Prev Loc V06-1	Possible Additional Specificity (not determined due to insufficien sequence data)	Revision Information/Remarks
17	H	Negative Control	Porcine *ACTA1*	516	P-2	**300**	CGCCATGTGTGACGAAGACGAGACC	+21			1H		
						301	CACGTACATGGCGGGCACGTTGAAG	+384					
	G	SLA-1*01XX(all)	SLA-1*0101~02/01rh28	209	P1023	**630**	CGGGGTCAGGGTCTCACACCTA	+347			5H	SLA-2*07rh12	Uncertain of SLA-12*ha03Q (Lr-28.0) due to primer mismatch at +518
						631	CCTCCTACGCTCCGCCACATT	+514					
	F	SLA-1*02XX(all)	SLA-1*0201~02/02we02	147	P1002	**602**	CAtTTCGTGCGGTTCGACAACT	+178		+159	5G		
						604	GGTGTTCAGGCCCACTCGGAG	+283					
	E	SLA-1*04XX(all)	SLA-1*0401/04gx01/04gz01/04we01	181	P1011	**614**	GCCtGACCGCGGGGACTCT	+123		+108	5F		
						615	GTAAGTCTGTGCGGTTTCCTTGACA	+261					
	D	SLA-1*05XX(all); SLA-1*an02	SLA-1*0501; SLA-1*an02	220	P1030D2	**737**	CCCCACTCCCTcAGCTATTTCTC	+89		+78		SLA-1*0102/01rh28	Similar to primer pair used in V06-1 at position 5E (P1032D1,+89/+266)
						641	GGAAAGTCTGTGAGGTGTCCCTTTG	+262	an02(+281,+284)				
	C	SLA-1*06XX(all); SLA-1*13XX	SLA-1*0601/06an04; SLA-1*1301	163	P1072D5	**524**	GCCTGACCKYGGGGACTCT	+123			5D	SLA-3*01rh12/01rh28; SLA-2*05rh03/05rh34	Degenerate bases added to forward primer (last used P1072D2)
						763	CTTGACTTTCCGCGTCTCCTC	+247					
	B	SLA-1*07XX(all)	SLA-1*0701~02	220	M1007	**382**	gCcGGGTCTCACACCATCCAGAT	+353		-2,-4 to exon 3	5C	SLA-2*07rh12	
						383	GGCCCTGCAGGTAGCTCCTCAAT	+528					
	A	SLA-1*08XX(all)	SLA-1*0801/08an03/08Lw02/08ms05/08pt13/ 08sk11/08sm08/08sy01	138	P1039D3	**648**	GtGGACTCCCGCTTCTTCATT	+135		+116	5B		Decreased both primers' Tm (last used P1039D2)
						684	CTCCCGATCCCAATACTCCG	+233					
28	H	SLA-1*09XX(all)	SLA-1*0901/09sm09	195	P1104D6	**908**	CCCACTCCCTGAGCTATTTCTT	+89			5A	SLA-1*0102/01rh28; SLA-2*07rh12	Increased both primers' Tm (last used P1104D5)
						671	TGATCTGTGTCTCCCGATCCCAATAG	+237					
	G	SLA-1*w10XX(all)	SLA-1*w10cs01/w10sm21	180	M1002	**530**	CTCCTCCTCCGCGGGTACGA	+404			6H	SLA-2*07rh12	
						531	CCACTCCACACACGTGCCCTC	+544					
	F	SLA-1*11XX(all)	SLA-1*1101~03/11mp11/11yn01	182	P1182D1	**436**	GTGTYCCGGCCCGACC	+112			6G		Decreased both primers' Tm and added degenerate bases (last used P1182)
						437	TGTGCGYTGCCCATGACAC	+260					
	E	SLA-1*12XX(all)	SLA-1*1201/12hy01/12Lw01	119	P1157	**742**	GTTCGACAGCGACGCCCTC	+186			6F		Decreased forward primer's Tm (last used P1142)
						733	GGTTAATCTGTGCGGTTTCCTTGA	+263					
	D	SLA-1*13XX(all)	SLA-1*1301/13ms21	211	P1055D3	**753**	CTCACACCCTCCAGAGCATGTTT	+360			6E	SLA-2*07rh12	
						104	CCCTGCAGGTAGCTCCTCCTA	+528					
	C	SLA-1*14XX(all)	SLA-1*1401	219	P1212D1	**909**	CCACTCCCTGAGCTATTTCTT	+89					
						788	GGTTAATCTGTGCGTTGTCCATGACA	+261					
	B	SLA-1*15XX(all); SLA-1*es11; SLA-2*01XX(all)	SLA-1*1501; SLA-1*es11; SLA-2*0101~02	253	P2079D2	**737**	CCCCACTCCCTcAGCTATTTCTC	+89*		+78*	3H	SLA-2*07rh12	
						401	TGTAGTAGCCGCGCAGGGTC	+300*					
	A	SLA-1*16XX(all)	SLA-1*1601	173	P1049	**653**	CGtGGACTCCCGCTTCTTCATT	+135		+116	6C	SLA-1*0102/01rh28	
						641	GGaAAGTCTGTGAGGTGTCCCTTTG	+262		+284			
39	H	SLA-1*16XX(all); SLA-1*an02; SLA-2*03XX(all); SLA-2*es22	SLA-1*1601; SLA-1*an02; SLA-2*0301~02/03gz01; SLA-2*es22	134	N2002	**242**	GCAGTTCGTGCGGTTCGACAGC	+175*			3F		
						243	CTCGGTAAGTCTGTGAGGTGTCCCTTTGTA	+260*	SLA-1*an02(+281); SLA-2*es22(+290,+292,+302)				
	G	SLA-1*cs02	SLA-1*cs02	208	P1174D5	**753**	CTCACACCCTCCAGAGCATGTTT	+360			6A	SLA-2*07rh12	
						762	TGCAGGTAGCTCCTCTCTCC	+526					
	F	SLA-1*rh03; SLA-2*es22	SLA-1*rh03; SLA-2*es22	193	P1172D3	**785**	GGTACAGWCAGTAYGSCTACGACA	+421*			6D	SLA-2*07rh12	
						752	TTCCCCATCTCCAGGTATCTGC	+569*					
	E	SLA-1*rh03; SLA-1*st11	SLA-1*rh03; SLA-1*st11	130	P1206D1	**730**	CGCaCCGAAaCCGAGGGA	+197		+183,+189			
						784	GCGCAGGTTGTTCAGGCT	+292					
	D	SLA-1*sk13	SLA-1*sk13	196	P1190D1	**431**	CAGGGTCTCACACCATCCAGAC	+353				SLA-2*07rh12	1) Also amplify with Hp-9.0 2) Decreased forward primer's Tm (last used P1190)
						768	GCCTCATGGGCCGCCTT	+505					
	C	SLA-3*01XX(all)	SLA-3*0101/01ev04/01rh12/01rh28	177	P3005D3	**861**	CTCCGCGGGTACAGTCAGTTT	+411				SLA-2*07rh12	1) Watch for weak false positive band 2) Similar to primer pair used in V06-1 at position 1G (P3004,+411/+544)
						461	GAGCCACTCCACACACGC	+550	01rh12(+560)				
	B	SLA-3*03XX(all); SLA-3*08XX(all)	SLA-3*0301~04/03an02/03an04/03an05/03pt31; SLA-3*0801	183	P3030D3	**1**	TCYTCCTCCRCGGGTACCA	+404				SLA-1*an02; SLA-2*07rh12; SLA-3*03an02/03an04	Degenerate bases added to forward primer (last used P3030D2)
						897	GAGCCACTCCACACASGC	+550	03an05(+560)				
	A	SLA-2*15XX(all); SLA-3*04XX(all); SLA-3*hb06	SLA-2*1501; SLA-3*0401~02/04es32; SLA-3*hb06	192	P3046BU1	**675**	GGAAGCCCCGTTTCATCGAA	+135			1E	SLA-3*01rh12/01rh28/07rh34; SLA-2*05rh03/05rh34	1) Also amplify with Hp-2.0 2) Weak amplification with SLA-2*15XX 3) Negative with SLA-1*1101 due to three forward primer mismatches
						871	GCAGGTTTTTCAGGTTCACTCGGA	+284	hb06(+300,+302)				

Tray Pos Row	Tray Pos Col	Group Specificity	Allele Specificity	Product Size (bp)	Primer Set ID	Primer ID	Primer Sequence (5'→3') (Upper = forward primer; lower = reverse primer; underlined = intron sequence; lower case = intentional mismatch)	3' Pos	Minor Sequence Mismatch	Intentional Mismatch	Prev Loc V06-1	Possible Additional Specificity (not determined due to insufficien sequence data)	Revision Information/Remarks
4 10	H	SLA-3*05XX(all)	SLA-3*0501~03/05sw01	138	P3019D2	**817**	CGTGGAaGAtACGCAGTTCGTGT	+166		+150,+152	1D		Decreased reverse primer's Tm (last used P3019)
						464	GTCTGTGCGTTGTCCTTGCTGA	+260					
	G	SLA-3*06XX(all); SLA-3*07XX(all)	SLA-3*0601~02; SLA-3*070101~02/07Lw02/07rh34	187	P3127	**891**	CGACGTGGGRCCAGACT	+382				SLA-2*07rh12	1) Similar to primer pair used in V06-1 at position 2B (P3121,+382/+534) 2) Degenerate base added to forward primer
						804	CGCGCCCTCCAGGTAGCTT	+534					
	F	SLA-3*07XX(all)	SLA-3*070101~02/07Lw02/07rh34	152	P3113U3	**888**	CGACCGCaGGAAGCCCCGT	+126		+124	1A		
						331	CCTCATCCCAATACTCCTGCCA	+238'					
	E	SLA-3*06XX	SLA-3*0601	152	P3057U2	**891**	CGACGTGGG**R**CCAGACT	+382			1C	SLA-2*07rh12	
						850	CATCGGCCGCCTCCCA	+502					
	D	SLA-3*06XX	SLA-3*0602	139	P3059D1	**851**	GCaGGAAGCCCCGTTTCAC	+131		+115	1B		Decreased reverse primer's Tm (last used P3059)
						532	TCCTCATCCCAATACTCCTGCCT	+229					
	C	SLA-1*11XX; SLA-3*03XX; SLA-3*04XX(all); SLA-3*hb06	SLA-1*1103; SLA-3*0301~04/03an02/03an04/03an05; SLA-3*0401~02/04es32; SLA-3*hb06	139	P3116	**560**	TCCCCACTCCCTGAGGTATTTCG	+88	SLA-1*1103/ SLA-3*0402/04es32(+81)		2E	SLA-1*0102/01rh28; SLA-2*05rh03/05rh34/07rh12	Also amplify with Hp-2.0
						825	CGGCTCCATCtGCGGATTG	+186	SLA-3*0303/0401(+204)	+194			
	B	SLA-2*01XX(all)	SLA-2*0101~02	172	P2003U2	**424**	CaCGACCGCGGGGAGC	+130		+116		SLA-2*07rh12	Watch for weak false positive band
						425	GTGCGCTGCCCATGACG	+270					
	A	SLA-2*02XX(all)	SLA-2*0201~02	138	P2008D1	**861**	CTCCGCGGGTACAGTCAGTTT	+420			3G	SLA-2*07rh12	Decreased forward primer's Tm (last used P2008)
						1006	CTGCTCCGCCACATTGGCT	+519					
5 11	H	SLA-2*03XX(all)	SLA-2*0301~02/03gz01	89	P2009U1	**1043**	AGTATTGGGATCGGGAGACRCAGATA	+270					Watch for weak false positive band/primer dimer
						1008	CTGGTTGTAGTAGtCGCGCAGGG	+311		+320			
	G	SLA-2*04XX(all)	SLA-2*0401/040201~02	311	N2003D1	**432**	AGGGAACCTGCGCACAGC	+314			3E		Decreased both primers' Tm (last used N2003)
						433	CACGTCGCAGCCGTACATGA	+362					
	F	SLA-2*05XX(all)	SLA-2*0501~03/05rh03/05rh34/05sy01	127	P2017	**1012**	CGGGCGCCGTGGATAGAGA	+232			3D		
						1029	CCTCGCTCTGGTTGTAGTAGCCAAG	+316					
	E	SLA-2*06XX(all)	SLA-2*0601~02/06an03/06me01/06sv01	125	P2020U1	**1016**	CGCCCCGAATCCGAGGAAA	+207			3C		1) Weak amplification 2) Degenerate bases added to reverse primer (last used P2020)
						438	GGKTGTTCAGGYYCMCTCGGTA	+292					
	D	SLA-2*07XX(all)	SLA-2*0701/07an05/07rh12/07we01	199	P2077	**1049**	GTCAtGGTCTCACACCCTCCAGGT	+362		-1 to exon 3	3B	SLA-2*07rh12	
						1050	TCCCTCCGCCACATTGGCT	+519	07an05(+537)				
	C	SLA-2*w08XX(all)	SLA-2*w08gx01/w08hy01/w08sw01	126	P2025D1	**1016**	CGCCCCGAATCCGAGGAAA	+207			3A		Degenerate bases added to reverse primer (last used P2025)
						435	GGTSTTCAGGYCCACTCGGTT	+292					
	B	SLA-2*w09XX(all)	SLA-2*w09an02/w09pt22/w09sn01	177	P2032D2	**434**	TGGGACCAGACGGGCTCT	+397			4H	SLA-2*07rh12	1) Watch for weak false positive band 2) Decreased both primers' Tm (last used P2032)
						520	CCTGCAGGTAGCTCCTCCAG	+537					
	A	SLA-2*10XX(all)	SLA-2*1001/10an01/10es21/10sk21/10sm01	104	P2074	**546**	AATCTCCGCAGATTCCAAAGATGC	+4				SLA-1*0102/01rh28/rh03; SLA-3*1rh12/01rh28/07rh34; SLA-2*05rh03/05rh34/07rh12	Similar to primer pair used in V06-1 at position 4G (P2072,-11/+66)
						1045	CCCGCACTCACCCGCCTGA	+66					
6 12	H	SLA-2*11XX(all)	SLA-2*110101~02/11sc01	123	P2041D2	**751**	GCCCCGAATCCGAGGGA	+206			4F		1) Decreased forward primer's Tm (last used P2041U1) 2) Negative with SLA-2*jh02 (Lr-59.0) due to seven reverse primer mismatches
						1027	TGTGCGCAGGTaCCCTCTGTAAA	+290		+301			
	G	SLA-1*es11; SLA-2*12XX(all)	SLA-1*es11; SLA-2*1201/12Lw01	160	P2046	**818**	CCTCCGCGGGTACAGTCAGTTC	+411*	SLA-1*es11(+403-404)		4E	SLA-2*07rh12	1) Replaced with primer pair previously used in V001-01 (last used P2046D3) 2) Strong with SLA-2*12XX; weak with SLA-1*es11
						1021	GCCTTGCAGGTAGCTCCTCCAG	+528*		+546*			
	F	SLA-1*11XX; SLA-2*w13XX(all)	SLA-1*1103; SLA-2*w13sm20	117	P2054U1	**1099**	TMGARMAGGAGGGGCAGGG	+236*			4D		
						1036	CGGgCTCGCTCTGGTTGTAGTA	+313*		+331*			
	E	SLA-1*09XX(all); SLA-2*w14XX(all); SLA-2*16XX(all) SLA-2*jh02	SLA-1*0901/09sm09; SLA-2*w14yn01; SLA-2*1601; SLA-2*jh02	131	P2174	**268**	GACGCTCCGAATCCGAGGGA	+197*		+183*			
						148	CGCAGGKTSTTCAGGCC	+292*	SLA-2*w14yn01(+311)				
	D	SLA-2*15XX(all); SLA-2*es22	SLA-2*1501; SLA-2*es22	90	P2137U1	**452**	GGACCGCGGCGGACACT	+486				SLA-2*07rh12	1) Watch for weak false positive band/primer dimer 2) Replaced previously used SLA-2*15XX primer pair (P2140D1) due to ambiguity with SLA-3*08XX
						236	GGCCCTGCAGGTAGCTCCTCCA	+538					
	C	SLA-2*06XX; SLA-2*w09XX; SLA-2*16XX(all)	SLA-2*0601~02/06me01; SLA-2*w09an02/w09sn01; SLA-2*1601	196	P2180	**190**	CCGCTTCCTCACCGTCGGGT	+151					
						316	GTAGTAGCCGCGCAGGGTG	+309					
	B	SLA-1*07XX(all); SLA-2*02XX(all); SLA-2*16XX(all); SLA-2*an04	SLA-1*0701~02; SLA-2*0201~02; SLA-2*1601; SLA-2*an04	138	P2181D1	**751**	GCCCCGAATCCGAGGGA	+197*					Replaced previously used SLA-2*an04 primer pair (P2062) due to potential false negative reaction
						316	GTAGTAGCCGCGCAGGGTG	+300*	SLA-2*02XX(+319)				
	A	SLA-2*jh02	SLA-2*jh02	175	P2170	**1068**	CGtGGACTCCCGCTTCCTCA	+142		+125			Negative with SLA-1*rh03 (Lr-21.0)
						364	TCGGTAAGTCTGTGCGGTTTCCTTGTAA	+270	+295				

Tray Pos Row	Tray Pos Col	Group Specificity	Allele Specificity	Product Size (bp)	Primer Set ID	Primer ID	Primer Sequence (5'→3') (Upper = forward primer; lower = reverse primer; underlined = intron sequence; lower case = intentional mismatch)	3' Pos	Minor Sequence Mismatch	Intentional Mismatch	Prev Loc V02-5	Revision Information/Remarks
17	H	Negative Control	Porcine *ACTA1*	516	P-2	**300**	CGCCATGTGTGACGAAGACGAGACC	+21			1H	
						301	CACGTACATGGCGGGCACGTTGAAG	+384				
	G	DRB1*01XX(all); DRB1*be01/ha01/ha04/Lu02	0101~02; be01/ha01/ha04/Lu02	162	PRB003	**1205**	CAGAAGCAGTACTATAACGGAGAGGAGC	+196	ha04/Lu02(+169,+173)			Decreased reverse primer's Tm (last used PRB003U1)
						1206	GTTGTGTCTGCAGTACGTGTCCACCG	+308	be01(+319-321); Lu02(+317)			
	F	DRB1*01XX(all); DRB1*be01/ha04/me02	0101~02; be01/ha04/me02	203	PRB098	**1513**	CGCATTTCTTGTTTCTGGTGAAAGA	+125				
						1515	CCGCATCTGCTCCAGGAGG	+285	me02(+309)			
	E	DRB1*02XX(all)	0201/0201br05/0201du02/02du01/02du03/02ka05/ 02ka06/02ka08/02sp02/02sp08/02zs13	115	PRB085	**1207**	GCATTTCTTGCACCTGTTGAAATTC	+126	02ka05(+112-113); 02ka08(+116); 02sp02(+112)			Decreased reverse primer's Tm (last used PRB085U1)
						1294	GTCtCTGTCGAAGCGCWRGAA	+196		+213		
	D	DRB1*03XX(all)	0301	180	GR006	**210**	CGGGTGAGGTTATTGCAGAGGAATTG	+179			4F	
						211	GTTGTGTCTGCAGTACGTGTCCACCG	+308				
	C	DRB1*04XX(all)	0401~04/04ga01/04ta01	206	SR008D2	**442**	CGCATTTCTTGTTTCTGSGGAAGG	+124			4E	
						443	CGCCCGCTTCTGCTCCAT	+289				
	B	DRB1*05XX(all)	0501~02/05ch01/05ka01/05ka03/05np01/05sp06	172	PRB016U2	**1297**	GGACGGAGCGGGYGCT	+161			4D	
						227	GTGTCCACTGAGGCCCGTGAGTC	+295	05np01(+303)			
	A	DRB1*06XX(all)	0601~03Q/06sL47/06zs12	122	PRB023D1	**1298**	GGGTGAGGTATCTGCTGAAGTACTTG	+180	0602~03Q/06zs12(+170); 06sL47(+162,+170)		4C	
						1299	GCTGTTCCAGTACTTGGCGTCT	+255	06zs12(+265)			
28	H	DRB1*07XX(all)	0701/07ka03/07yo02	133	PRB026	**224**	GGACCGAGCGGGTGAGGTTCA	+166				
						1227	TGGCTGTTCCAGTACTTGGCTGAA	+255				
	G	DRB1*08XX(all); DRB1*ka04/ka05/oj01	0801/0801hg06/08hg09/08ka83/08ka92/08sp05; ka04/ka05/oj01	108	PRB111	**1521**	CGCAGCGCATTTCTTGTACCWGT	+118	08ka83(+112-113); 08ka92/08sp05(+112); ka04(+112)			
						1522	CGCACGTACTCCTCTCCGTTATAGTAC	+177	08hg09(+198)			
	F	DRB1*09XX(all); DRB1*du05/La02/oj02	0901/0901br04/09sL48/09ta01; du05/La02/oj02	105	PRB108	**1285**	CAGCGCATTTCTTGTTTCTGGG	+119	La02(+109)			Similar to previously used SR002 (+119/179) in position 5G of V02-5
						1520	CGCACGAACTCCTCTCCGTTATAGTAT	+177				
	E	DRB1*09XX(all); DRB1*ka09/kb02/La02/La04	0901/0901br04/09sL48/09ta01; ka09/kb02/La02/La04	157	PRB034D3	**1280**	TTCTTCAATGGGACCGAGCA	+155	La04(+150)		5F	Watch for primer dimer; not sure du05(mismatches at +152,+280)
						490	CCAGGAGGTCCTTCTGGCTGTTAT	+269				
	D	DRB1*10XX(all); DRB1*er01/La03	1001/10jh01/10ka06/10Lu03/10sp07; er01/La03	135	PRB037U1	**1506**	ACGCAGCGCATTTCTTCTTTATGGA	+119			5E	
						1236	GGTACTCGCCCACGTCGCTA	+210	er01(+224)			
	C	DRB1*11XX(all)	1101~02/11ac21/11br02/11sp01/11zs10	109	PRB042D2	**1216**	CGAGTTCtGGGAAGTGACCGAAT	+244		+229	5D	
						1287	GTGTCTGCAGTACGTGTCCACTG	+308				
	B	DRB1*w12XX(all)	w12ka02/w12ka05/w12ka12	186	PRB048D1	**1246**	GACGGAGCGGGTGAGGTTTC	+166	w12ka05(+157)		5C	
						1218	TTGTGTATGCAGTACGTGTCCACTGAG	+306				
	A	DRB1*13XX(all)	1301	182	GR016	**228**	TGGAGAGGCAGTACTATAACGGAGAGGAAC	+196			6A	
						229	ATCCAAGATCCTGTAGTTGTGTCTGCACAC	+319				
39	H	DRB1*14XX(all)	1401	113	PRB056	**1246**	GACGGAGCGGGTGAGGTTTC	+166			6F	
						1251	CGTCTGGCCGCCCCAGT	+243				
	G	DRB1*ka13	ka13	160	PRB107	**1207**	GCATTTCTTGCACCTGTTGAAATTC	+126	ka13(+116)			
						487	GtCGTCTGGCCGCCCAAAT	+243		+260		
	F	DRB1*ka14	ka14	134	PRB053U1	**1248**	AACACGAGTGTCATTTCTTCACTGGA	+147			6E	
						1530	AGGCCGCCCCAGCTCG	+240				
	E	DRB1*kb02/kb03N/kb04N	kb02/kb03N/kb04N	202	PRB092D1	**1509**	GCATTTCTTGCTTCTGGTGAAACA	+125	kb02(+112)			
						1508	CCGCCTCTGCTCCAGGAG	+286				
	D	DRB1*La03/La04/La05	La03/La04/La05	197	PRB090	**1506**	ACGCAGCGCATTTCTTCTTTATGGA	+119				Watch for primer dimer
						1232	CAGGAGGTCCTTCTGGCTGTTATAGTT	+265				
	C	DRB1*04XX; DRB1*11XX; DRB1*ka13	0403~04; 1101/11ac21; ka13	117	PRB063U3	**1290**	ACGGAGCGGGTGAGGTTTC	+166			6H	
						215	CTTGtCGTCTGGCCGCCCAAAT	+243	1101(+262)	+260		
	B	DRB1*04XX	0401~02/04ga01/04ta01	160	SR006U1	**485**	CGGAGCGGGTGCGGTTG	+165				Increased reverse primer's Tm (last used SR006)
						1292	TCCGCCCGCTTCTGCTCCAT	+289				
	A	DRB1*04XX	0401~02/04ga01/04ta01	118	SR007	**486**	CGGGACGGAGCGGGTGC	+160			5A	Watch for primer dimer; may not amplify 0402/04ga01 due to mismatches at +255-258
						487	GtCGTCTGGCCGCCCAAAT	+243	0402/04ga01(+255-258)	+260		

Tray Pos Row	Tray Pos Col	Group Specificity	Allele Specificity	Product Size (bp)	Primer Set ID	Primer ID	Primer Sequence (5'→3') (Upper = forward primer; lower = reverse primer; underlined = intron sequence; lower case = intentional mismatch)	3' Pos	Minor Sequence Mismatch	Intentional Mismatch	Prev Loc V02-5	Revision Information/Remarks
4 10	H	DQB1*01XX(all)	0101/01be01/01ha01/01Lu01/01me03/01sh01	165	PQB012D4	**472**	GATACATCTACAACCAGGAGGAGCTT	+207			1G	
						1380	GGTAGTTGTGTTTGCACACCG	+326				
	G	DQB1*01XX(all); DQB1*sh03	0101/01be01/01ha01/01Lu01/01me03/01sh01; sh03	180	PQB014	**1313**	GCAGAGGATTTCGTGTACCAGTTTAAGTT	+134	0101/01be01/01ha02/01Lu01(+122); 01me03(+122-123)			
						1315	GtCGTTCCAGGAGTCGGCCT	+266		+284		
	F	DQB1*02XX(all)	0201~04/02du01/02kg02/02La03/02me01/02zs16	146	PQB015U2	**1373**	GGGGCGTGGCCAGGTGGG	+187	02La03(+170); 02zs16(+172-173,+181)		1F	
						1316	GGaCCGCTTCTGCTCCAGGAC	+295	0203/02zs16(+308)	+313		
	E	DQB1*03XX(all)	0301~03	166	PQB018U1	**1345**	TTCCAGTTTAAGGGCGAGTGCTACTTCTA	+149	0302(+122); 0303(+121)			
						1346	GGCTGTTCCAGGAGTCGGCCT	+266				
	D	DQB1*04XX(all)	040101~02/0402/0402we01/04hg09/04sk51/04sp16	197	MQ005	**147**	CAGCGGGTGTGGAGCGTGGA	+179				
						161	TCCTCTATCTGGTAGTTGTGTTTGCACACA	+327				
	C	DQB1*05XX(all)	0501~03/05sp06	193	PQB051D3	**1326**	GCAGCGGGTGCGGCTCT	+175			1C	Watch for false positive band or primer dimer
						1391	TATCTGGTAGTTGTGTTTGCACACC	+327	0503(+349-350)			
	B	DQB1*06XX(all); DQB1*zs12	0601~02/06sp01; zs12	204	PQB092D1	**1305**	ACtCAGCGGGTGCGGCA	+173		+159		
						153	GCCTTCCTCTATCTGGTAGTTGTGTTTGC	+332				
	A	DQB1*07XX(all)	0701	154	PQB031U1	**1339**	CACGTGCGCTTCGACAGCA	+223			1A	
						1340	CTTCCTCTATCTGGTAGTTaTGTTTGCACACA	+327		+339		
5 11	H	DQB1*08XX(all); DQB1*zs13	0801/08ch01/08Lu03; zs13	148	PQB009D9	**1356**	CATCTACAACCAGGAGGAGTACGC	+209	zs13(+186-187)		2H	Decreased forward primer's Tm (last used PQB009D8)
						275	GCACAtCGTGTCCAGCTCT	+315		+328		
	G	DQB1*09XX(all)	0901/09zh01	146	PQB033D2	**1395**	TGCGGCTCGTGACCAGATT	+185				Slightly weak; decreased both primers' Tm (last used PQB033D1)
						1396	CCGCGTCTGCTCCAGGAT	+295	09zh01(+306)			
	F	DQB1*09XX(all); DQB1*Lu02	0901/09zh01; Lu02	180	PQB035D4	**1395**	TGCGGCTCGTGACCAGATT	+185	Lu02(+172-173)			Decreased forward primer's Tm (last used PQB035D3)
						1380	GGTAGTTGTGTTTGCACACCG	+326				
	E	DQB1*es51; DQB1*zs14	es51; zs14	161	PQB087	**1356**	CATCTACAACCAGGAGGAGTACGC	+209				
						254	GGTAGTTGTGTTTGCACACCSTGTCCAC	+319				
	D	DQB1*02XX; DQB1*Lu02; DQB1*zs13	0201/0204/02du01/02kg02/02me01; Lu02; zs13	193	PQB052U3	**1377**	AACtCAGCGGGTGCGGGGC	+174		+159	2E	Not sure 02La03 (mismatches at +170);increased reverse primer's Tm (last used PQB052U2)
						1397	CTGGTAGTTGTGTTTGCACACCG	+326				
	C	DQB1*02XX	0202/02zs16	176	GQ002D3	**1348**	CCAGGTGGGTCTACAACCG	+197	02zs16(+181)		2D	
						1350	CTCTATCTGGTAGTTGTGTTTGCACACC	+327				
	B	DQB1*02XX	0201/0203/02du01/02kg02/02me01	133	PQB002U3	**96**	AAtGCAGCGGGTGCGGGG	+173		+158	2C	Not sure 02La03 (mismatches at +170);increased reverse primer's Tm (last used PQB002U2)
						1375	CTGGtCGTTCCAGTAGTCGGCGGT	+265		+284		
	A	DQB1*02XX; DQB1*03XX	0203/02zs16; 0301	165	PQB044U1	**1345**	TTCCAGTTTAAGGGCGAGTGCTACTTCTA	+149	0203(+122)			Weak with DQB1*0301
						169	TGTCCASCTCGGCCCGCA	+308				
6 12	H	DQB1*02XX; DQB1*zs13	0202/0204; zs13	173	GQ003D7	**244**	GCGGSKCGTGGCCAGG	+183				Weak
						426	TGTGTTTGCACACCGTGTCCAG	+319				
	G	DQA*01XX(all)	0101~03/01ch01/01my01	141	PQA004D1	**1148**	ACTGTGTTTTCCAAGTCTCCAGTGATA	+372				Decreased forward primer's Tm (last used PQA004)
						1107	GAAGCTGGTCTCAGAAAAACCTTTGA	+461				
	F	DQA*02XX(all); DQA*ka01	0201~05/0201~05/02cs01/02xu01; ka01	210	PQA025U1	**1101**	GGCTTAAATGTCTACCAGTCTTACGGTC	+130				Degenerate base added to reverse primer (last used PQA025)
						1157	GTTGGAACGTTTAATCAGGRTGTTCAAA	+285	02xu01(+306)			
	E	DQA*03XX(all)	0301/03ta01/03we01	160	PQA009D3	**1159**	TGATGGCGACGAGGAATTCTAT	+177				Decreased both primers' Tm (last used PQA009D3)
						1160	GTTGTTGGAGCGTTTAGTCACA	+294				
	D	DQA*04XX(all)	0401/04ta01	124	PQA014	**1118**	CTGGAGAAGAAGGAGACTGTCTGGA	+208				
						1119	AGCGTTTAGTCAGGATGTTCAAGGTAA	+281				
	C	DQA*04XX(all)	0401/04ta01	148	PQA013D2	**1116**	CCATGAATTTGATGGTGACGAGC	+169				Decreased reverse primer's Tm (last used PQA013D1)
						1152	GATGTTCAAGGTAAGTTTTCCCAC	+271				
	B	DQA*w05XX(all)	w05ch01	111	PQA019D2	**1109**	GGAGAAGAAGGAGACTGTCTGGCA	+209				Decreased reverse primer's Tm (last used PQA019)
						1152	GATGTTCAAGGTAAGTTTTCCCAC	+271				
	A	DQA*ka01	ka01	120	PQA023D1	**1136**	GAAGGAGACTGTCTGGCAGTTGAC	+215				
						1124	TTGGAACGTTTAATCAGGATGTTCAAA	+285				

7.4 Figures and Tables

7.4.1 List of Figures

7.4.2 List of Tables