Safety of xenotransplantation: Development of screening methods and testing for porcine viruses

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Elena Janich

(geb. Plotzki)

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1. **Gutachter: Dr. Joachim Denner**

   Robert Koch Institut
   Fachgebiet 18 „ HIV und andere Retroviren“
   Nordufer 20, 13353 Berlin

2. **Gutachter: Prof. Dr. Rupert Mutzel**

   Freie Universität Berlin
   Institut für Biologie – Mikrobiologie
   Königin-Luise-Straße 12 – 16, 14195 Berlin

Disputation am:_____

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Abbreviations

AHXR	acute humoral xenotransplant rejection
AIDS	acquired immune deficiency syndrome
AvHV-1	alcelaphine herpesvirus-1
BCMV	baboon cytomegalovirus
CD46	cluster of differentiation 46
CMAH	cytidine monophosphate-N-acetylneuraminic acid hydroxylase
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats
dpf	designated-pathogen free
EBV	Epstein Barr virus
EPCR	endothelial protein C receptor
ERV	endogenous retroviruses
GGTA1	alpha-galactosyltransferase 1
HAR	hyperacute rejection
HCMV	human cytomegalovirus
HHV-8	human herpesvirus 8
HIV	human immunodeficiency virus
HO-1	heme oxygenase 1
HTLV	human T-cell lymphotropic virus
KO	knockout
KSHV	Kaposi sarcoma-associated herpesvirus
Neu5Gc	2 N-glycolylneuraminic acid-terminated gangliosides
NHP	non-human primates
OvHV-1	ovine herpesvirus-1
PBMCs	peripheral blood mononuclear cells
PERV	porcine endogenous retrovirus
PRRSV	porcine reproductive and respiratory syndrome virus
PTLD	post-transplant prolipharative disorder
SARS	severe-acute-respiratory-syndrome virus
SIV	simian immunodeficiency virus
SIVcpz	SIV of chimpanzee
SIVsm	SIV of sooty mangabey
spf	specified-pathogen free
STLV	simian T-cell leukemia virus

SV 40	simian virus 40
TALEN	transcription activator-like effector nucleases
TFPI	tissue factor pathway inhibitor
TM	thrombomodulin
ZFN	zinc finger nuclease
αGal	α1,3-galactosyl-galactose

Summary

Xenotransplantation using pig cells, tissues or organs might be a promising solution to overcome the shortage for organs suitable for allotransplantation. Because of several reasons, the pig is currently the favoured donor species. However, the use of porcine xenotransplants is associated with the risk of transmitting porcine viruses to the human xenotransplant recipient. Among them porcine endogenous retroviruses (PERVs), porcine cytomegalovirus (PCMV), porcine lymphotropic herpesviruses (PLHVs), porcine circovirus 2 (PCV2) and hepatitis E virus (HEV) play a role. Some of them cause immunosuppression and a zoonotic potential of others has been supposed. Therefore the possibility of direct transmission of those viruses between pigs and humans might be possible. Strategies to avoid the transmission of those pathogens are currently of main importance to increase lifetime of the transplant and therefore to save many lives of people standing on the transplant waiting list. To select virus-free animals as putative donor pigs and to recognise transmission of pathogens to transplant recipients, sensitive detection methods are needed.

In this study the prevalence and expression of these selected viruses should be investigated and assessed in order to obtain safe and healthy donor pigs for xenotransplantation studies. Therefore highly sensitive PCR-based methods, real-time PCR and real-time RT-PCR specific for all the viruses listed above, as well as immunological methods measuring virus-specific antibodies by Western blot analysis or ELISA were developed. Recombinant viral proteins were cloned, expressed and chromatographically purified as well as purified virus particles were expanded to be used as antigens.

The methods were developed and optimized to screen (i) Göttingen minipigs, a well characterized pig breed which is kept in a specific-pathogen free facility, (ii) Aachen minipigs, a pig breed existing since 2013, (iii) slaughterhouse pigs from a butchery in the north of Berlin and (iv) multiply genetically modified pigs produced especially for xenotransplantation.

Human-tropic PERV-A and PERV-B were found in all pigs and pig-tropic PERV-C and recombinant PERV-A/C were found in many pigs. HEV, PCMV, PLHVs and PCV2 were found in a few animals. No transmission of the porcine viruses listed above was observed during the transplantation of genetically modified islet cells into four marmosets. However, when transgenic pig hearts were transplanted into baboons, then PCMV and HEV were found transmitted, despite the fact that the donor pigs were negative when testing blood and antibody response. To avoid future transmissions of porcine viruses, more sensitive detection methods, different time points of testing, and different source materials, including oral and anal swabs, should be used.

In the study sensitive and reliable methods for the detection of porcine viruses were developed and those viruses were detected in all tested pig herds. Furthermore, potentially zoonotic viruses like HEV and viruses causing immunosuppression like PCMV, PLHVs and PCV2 are present in pigs for slaughter. Although the expression of these viruses were low, the meat-producing and -processing industry should be aware of the improvement of hygienic standards. The newly developed detection methods are a prerequisite for the selection of virus-free pigs for transplantation trials as well as elimination programs based on treatment, vaccination, Caesarean delivery, early weaning and embryo transfer.

Zusammenfassung

Die Transplantation von porzinen Zellen, Gewebe oder Organen stellt eine erfolgsversprechende Methode dar, um den Mangel an Organspendern auszugleichen und somit das Leben und die Lebensqualität vieler kranker Menschen zu verbessern und zu retten. Aufgrund verschiedener Aspekte wird hierfür das Schwein als Spendertier favorisiert.

Jedoch besteht das erhöhte Risiko, dass Mikroorganismen während der Transplantation, begünstigt zum Beispiel durch eine Therapie mit Immunsuppressiva, auf den Menschen übertragen werden und somit eine Bedrohung für den Xenotransplantat-Empfänger darstellen können. Einige dieser Viren sind in der Lage, eine zusätzliche Immunsuppression hervorzurufen und somit den Ausbruch von Sekundärerkrankungen zu begünstigen. Andere stehen durch ihre enge genetische Verwandtschaft zwischen Mensch und Tier im Verdacht, ein zoonotisches Potential zu besitzen. Dabei spielen vor allem die porzinen endogenen Retroviren (PERV), das porzine Cytomegalievirus (PCMV), die porzinen lymphotropen Herpesviren (PLHV), das porzine Circovirus 2 (PCV2) und das Hepatitis E virus (HEV) eine Rolle. Für die Xenotransplantation ist es wichtig, Strategien zu entwickeln, die die Pathogenübertragung verhindern, um die Funktion und Qualität des Transplantats zu erhalten. Um virus-freie Donorschweine für die Xenotransplantation auszuwählen und die Übertragung auf den Transplantatrezipienten zu erkennen, sind sensitive und selektive Detektionsmethoden notwendig.

In dieser Studie soll die Prävalenz und Expression der ausgewählten porzinen Viren im Hinblick auf die Auswahl von sicheren, gesunden Donortieren untersucht und beurteilt werden. Dafür wurden hoch-sensitive PCR-basierte Methoden, Real-time PCRs, RT-Real-time PCR sowie immunologische Methoden wie Western Blot und ELISA, die die Antikörperantwort auf ein spezifisches Virus detektieren, neu entwickelt, verbessert und etabliert. Rekombinante virus-spezifische Proteine wurden kloniert, exprimiert, chromatographisch aufgereinigt und außerdem wurden virale Partikel produziert. Diese Methoden wurden verwendet, um die (i) Göttinger Minipigs, die (ii) Aachen minipigs (iii) Schlachttiere, sowie (iv) multitransgene Schweine, speziell gezüchtet für die Xenotransplantation, zu analysieren.

PERV-A und-B konnte in allen Schweinen und das PERV-C Virus sowie die Rekombinante PERV-A/C in vielen Tieren detektiert werden. HEV, PCMV, PLHVs und PCV2 wurden in vielen Tieren gefunden. Während der Inselzelltransplantation von multitransgenen Schweinen in vier Seidenaffen, konnte keine Übertragung der untersuchten Viren gefunden werden. Jedoch wurde bei der Transplantation von multitransgenen Herzen PCMV und HEV übertragen, obwohl die Donortiere zuvor negativ per PCR und Antikörperantwort getestet wurden. Um zukünftig eine sichere Xenotransplantation zu gewährleisten, ist es notwendig, dass noch

sensitivere Methoden etabliert, verschiedene Zeitpunkte der Testung gewählt werden und auch verschiedenes Material zur Verfügung steht, wie beispielsweise Mund-oder Afterabstriche.

Zusammenfassend konnten während dieser Studie sensitive und zuverlässige Methoden etabliert und die oben gelisteten Viren in fast allen Schweinerassen detektiert werden. Zusätzlich wurden in Schlachttieren Viren nachgewiesen, die eine Immunsuppression hervorrufen können, sowie HEV, welches zoonotisches Potential besitzt. Obwohl die Expression dieser Viren gering war, sollte sich die Fleischindustrie dieser Problematik bewusst sein und ihre Hygienemaßnahmen danach ausrichten. Die neu entwickelten Methoden sind notwendig für die Auswahl von virus-freien Tieren für Transplantationsstudien sowie Eliminierungsprogramme basierend auf medikamentöser Therapie, Vakzinierung, Kaiserschnitt, frühes Absetzen vom Muttertier und Embryonentransfer.

1 Introduction

1.1 Transplantation

The transfer of living cells, tissues or organs from one individual to another from the same species is called allotransplantation and seems to be the most ideal solution for patients suffering from organ failure. Nevertheless, this field remains limited by donor organs. In 2015 Eurotransplant was able to allocate 7.145 organs from 2063 deceased donors to the patients, but there are still 14.560 people on the active waiting list for transplantations (Figure 1)[2]. The gap existing between the people on the waiting list and organs available for transplantation is even bigger, because the waiting list only contains the number of patients who actually have a life threatening indication. In addition to the patients whose lives are directly threatened, there are candidates for organ transplantations whose quality of life could be remarkably improved[3]. These patients are, for example people suffering from diabetes, Parkinson's disease or chronic kidney disease. To overcome the discrepancy that the demand for human organs far exceeds the supply, different alternatives for allotransplantation are under investigation. One approach is the use of artificial organs (tissue engineering) or creating vascularized organs from stem cells. However, this field so far offered limited success and the use of pluripotent stem cells to grow new organs remains highly controversial[4]. Another way to overcome this problem could be the use of organs from other species that is called xenotransplantation[2].

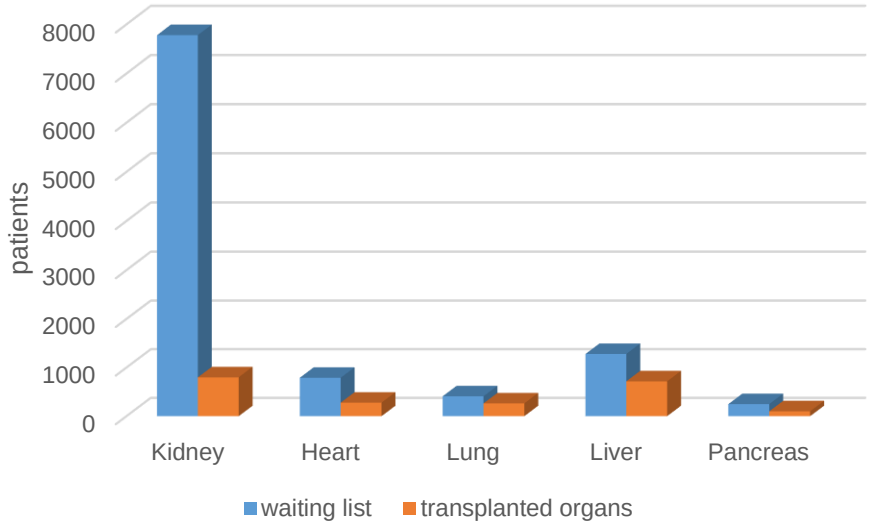

Figure 1 Transplantations performed and waiting list for organs in Germany in 2015
In 2015 there were 10238 people on the waiting list for a new donor organ. Only 863 transplantations were performed during the year. 7781 people were waiting for a new kidney, but only 799 transplantations could be performed. 790 patients were waiting for a new heart, however, only 278 people got a new transplant. 409 people needed a new lung and 263 transplantations were performed. Only 717 patients of 1280 people on the waiting list got a new liver and 101 pancreas were transplanted instead of 248 which were actually needed. [2]

1.2 Xenotransplantation

Xenotransplantation is defined to be any procedure that involves the transplantation, implantation, or infusion of fluids, cells, tissues or organs from one species to another including non-living or acellular biomaterials (heart valves, blood vessels, tendons) of non-human species[5]. More specifically it is understood to be the transplantation of animal cells, tissues or organs, mainly from pigs to humans. There are considerable advantages of xenotransplantation towards allotransplantation: an inexhaustible source of cells[6], the disappearance of long waiting times and better planning for transplantations as well as the reduced risk of infection through periodically control of donor animals throughout life[7], and last but not least the prevention of illegal organ trade[8]. The disadvantages include theological, ethical and legal reservations together with three major problems: the guarantee of the anatomic-physiological compatibility for the functionality of the organ, the prevention of the immunological rejection of the organ and the microbiological safety to avoid the transmission of potentially pathogenic microorganisms which may induce zoonosis, within xenotransplantation named xenozoonosis [5].

Throughout the last decades first transplantations of livers, kidneys and hearts of primates (baboon, chimpanzees), sheep and pigs to humans have been performed[9]. In Poland and India two patients received a pig heart in 1992 and 1996[10] as well as the transplantation of two baboon livers[11] and a transplantation of a pig liver in a patient with liver failure. Nevertheless, these trials showed limited success[12]. Only one patient suffering from severe renal failure survived for nine months[13]. All other human patients receiving a xenotransplant succumbed within 70 days[9].

The rejection of the transplant which has not been understood so far, anatomic-physiological incompatibility and uncontrolled infections lead to functional loss of the transplant and death of the recipient. The unsatisfying results of xenotransplantation to humans show that big effort needs to be put in the understanding of xenorejection, the balance between immunosuppression and the risk of infection by pathogens on the way towards the clinic[14]. In contrast to the transplantation of whole organs, the use of cells and tissues seems to be more promising and successful. In the last years, implantation of pig embryonic cells into the brains with Parkinson´s disease[15] or Huntington´s disease[7], and the ex-vivo perfusion of pig organs or artificial organs which are utilized with porcine cells, have not shown any adverse effects, neither have they reduced symptoms nor improved life quality of patients[16]. Especially the transplantation of porcine islet-cells results in the most successful procedure in treating type I-diabetes[17].

The problems of rapid early transplant destruction or T-cell mediated rejection are being resolved by genetic engineering combined with immunosuppressive therapy. Results of pig-to-diabetic non-human primate islet xenotransplantation show insulin independence achieved for periods over one year[18]. An alternative possibility is the isolation of islets and encapsulation within a micro- or macro-device protecting them from the human recipient´s immune response and avoiding the diffusion of cells or microorganisms in the recipient by allowing transition of insulin, nutrients and glucose. With encapsulation no immunosuppressive drugs need to be taken, which is a major advantage. Clinical trials are currently underway[19].

1.2.1 Pigs are ideal donors for xenotransplantation

For various reasons only pigs (*Sus scrofa*) are chosen as suitable donor species for xenotransplantations in humans[20]. Early sexual maturity (4-8 months), short gestation time (115 days) and multiple births per litter (5-12 piglets) allow that they can be easily bred at low costs and, most importantly, under pathogen-controlled and hygienic conditions in specified or designated pathogen free-facilities (spf or dpf). Under spf/dpf-conditions putative pathogenic and zoonotic microorganisms could be eliminated. Other advantages using pigs as donor animals for xenotransplantation are the physiological and anatomical similarities to humans. There had already been a long history of providing medicinals like skin, insulin, cardiac prostheses, clotting factors[21]. Moreover, they can be used to manipulate their genes and generate multitransgenic pigs expressing, for example, human complement regulating genes or by the knockout of pig cell surface molecules [22]. Furthermore, the ethical doubts coming along with pigs as a source for donor organs are low in our society since millions of pigs (~ 60 million pigs in 2016 in Germany) are slaughtered for pork consumption every year[23].

For a while also non-human primates (NHP) were discussed as potential donor animals for xenotransplantation studies because they are most similar to humans, anatomically and physiologically. However, because of ethical doubts, potential virus transmission and the expensive rearing as well as the smaller size of organs seem to have abandoned hopes of using NHP as xenotransplant donors. In addition, the costs of raising pathogen-free herds in a large quantity to satisfy clinical demand would be an illusion [21] (Table 1). Nevertheless, one major problem is also the infectious risk for human patients and their contacts because of the phylogenetic relationship and similarity of the immune systems among primates. Some monkey viruses like human herpes virus 8 (HHV-8) are deadly to humans in a matter of days[24]. Over 20 potentially lethal viruses are known that could be transmitted from NHP to humans including Hepatitis A and B[25], Marburg virus [26], Ebola[27], herpes B, simian virus 40 (SV40)[28] and simian immunodeficiency virus (SIV), whereas the human immunodeficiency virus (HIV)

type -1 and -2 are a result of cross species transmission between the SIV of chimpanzee (SIVcpz) and the sooty mangabey (SIVsm) [29].

		non-human primates	
	pig	apes	monkeys
physiology	similar	almost identical	similar
transplant rejection	very strong	not so strong	strong
organ size	similar	almost identical	too small
gestation time (days)	100	251-289	170-193
numbers of offspring	10-18	1-2	1-2
spf-keeping	possible	currently not possible	currently not possible
cloning	possible	currently not possible	currently not possible
infectious risk	low, if spf-keeping possible	very high	very high
availability	unlimited	very limited	limited
costs	low	very high	high
species protection	animal protection	very strict protection	strict protection

Table 1 Advantages and disadvantages of different donor animals for xenotransplantation in humans [30]

Just as in allotransplantation, the immunological rejection of the xenotransplant is one of the most formidable obstacles to overcome and this is much more complex (Figure 2 part A). During the last xenotransplantations of whole organs performed from pigs to humans (1992 heart transplantation and 1994 liver transplantation) the recipients died within 24 hours because of the strong immune rejection of the transplant[12]. The use of pig xenotransplant lead to a discordant hyperacute response (hyperacute rejection, HAR) and a loss of the xenotransplant within a few minutes or hours[31]. In the pathogenesis of HAR, xenoreactive antibodies that bind to the endothelial lining of the transplant vasculature and the complement system appear to be of primary importance[32]. HAR is initiated by pre-existing antibodies against endothelial α1,3-galactosyl-galactose (αGal,GT) epitopes, resulting in complement activation and rapid transplant destruction[1]. Genetic inactivation of the alpha-galactosyltransferase 1 (GGTA1) gene, which is adding α-Gal epitopes to pig cell surface molecules[33], and the overexpression of human complement-regulatory genes such as CD46 (membrane cofactor protein)[34], CD55 (complement decay-accelerating factor) [35] and CD59 (protectin) could overcome this problem[36-37]. Another carbohydrate xenoantigen for pre-existing antibodies in human sera are 2 N-glycolylneuraminic acid-terminated (Neu5Gc)

gangliosides which are produced in pigs and other mammals as well as in NHPs[38]. Humans do not synthesize Neu5Gc, because a DNA mutation causes the lack of the enzyme cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) which is required for Neu5Gc production. A double knockout (KO) of both the GGTA1 and CMAH genes further reduces the xenoantigenicity of porcine organs in humans[39]. Moreover, it could be shown that T-cell responses are reduced by the knockout of GGTA1 xenotransplant recipients or simultaneous expression of CTLA4-IgG[40-41]. The overexpression of human PD-L1 triggering the inhibitory PD-1 receptor showed human T and B cell activation and elicited antibody response[42]. Several human genes, like human thrombomodulin (hTM)[43], heme oxygenase 1 (HO-1) and the tumor necrosis factor-induced human protein A20, as well as the tissue factor pathway inhibitor (TFPI) and endothelial protein C receptor (EPCR) have been explored to improve long-term survival of porcine xenotransplants after the transplantation in NHP[44]. The problem of using transgenic pigs is that their immune system is similar to the human immune system and therefore poses a risk of infection with human pathogens by, for example, animal care attendants or veterinarians. It is known that some of the integrated human complement-regulating factors serve as receptors for human viruses. The human CD46 molecule is a receptor for measles[45] and CD55 a receptor for enterovirus 70, a family member of the *Picornaviridae* [46].

Another immunological barrier to overcome is the acute humoral xenotransplant rejection (AHXR) or delayed xenotransplant rejection, which has poorly been examined until now[47]. This process takes place over a period of days and weeks and has been mainly characterized in heterotopic cardiac xenotransplantations[48]. It shows similar pathological effects like the HAR involving swelling, edema and vascular thrombosis[49]. In contrast to HAR it is caused by the binding of antibodies to the transplant with or without complement. Therefore AHXR cannot be prevented by complement inhibitors, whereas HAR can be treated in this way (Figure 2 part B).

Moreover, cellular rejection mediated by T-cells or chronic rejection might occur after xenotransplantation[50]. The successful overcoming of these barriers requires high concentrations of immunosuppressive drugs which already made it possible that in transplantations delayed xenotransplant rejection was studied, and even more important, xenotransplant survival could be prolonged[51]. This was, for example, shown in a discordant cardiac xenotransplantation in a pig-to-baboon model[51].

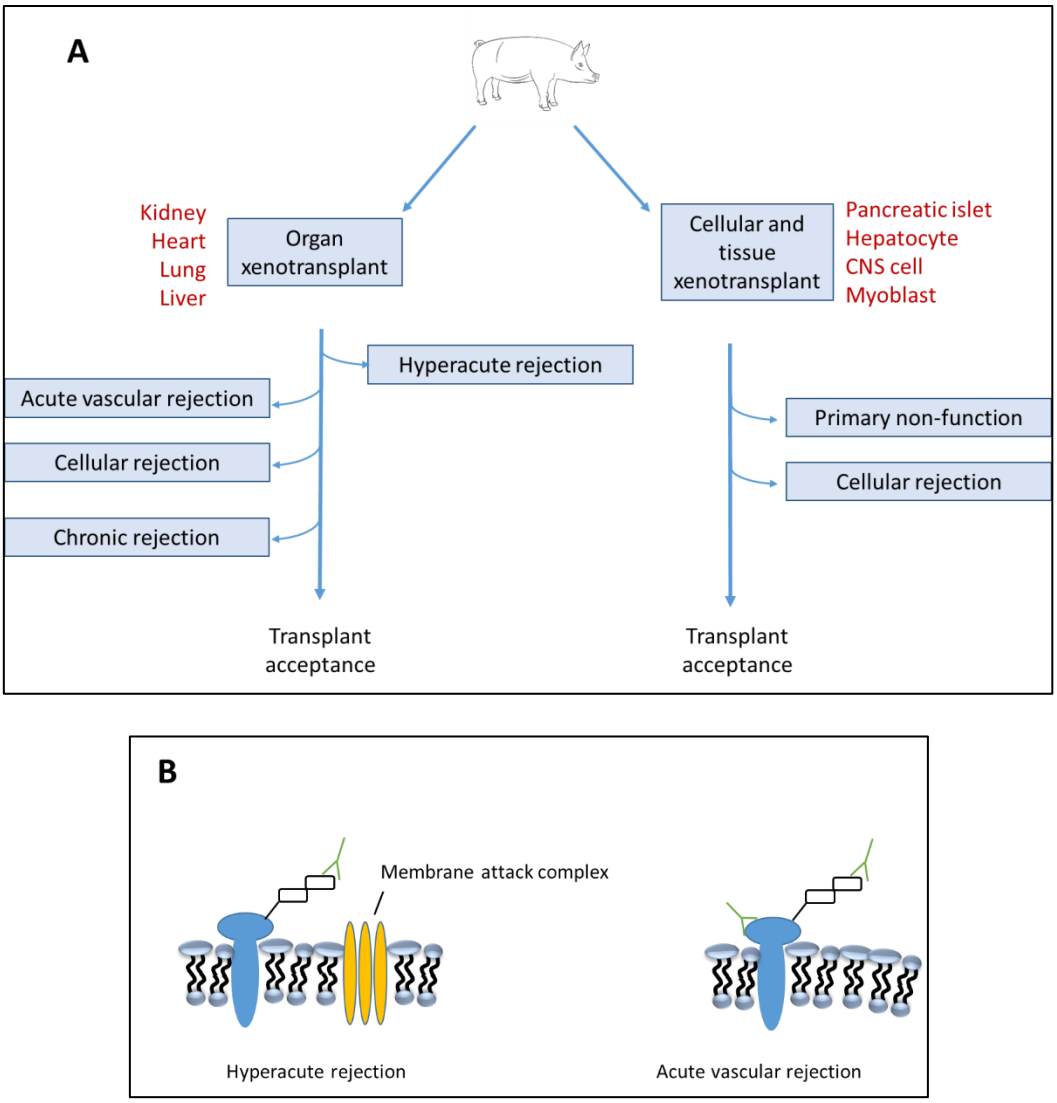

Figure 2 Types of xenotransplant rejection (modified from[1])

(A) Depending on the type of the transplant, different reactions occur. Organ xenotransplant have an influence on vascular rejection induced by antibodies and cellular rejection caused by T-cells, whereas cellular and tissue xenografts are defeated by primary non-function caused by macrophages and cellular rejection.

(B) α-1.3-Gal plays a role in hyperacute and acute vascular rejection. Hyperacute rejection occurs by the binding of large amounts of antibodies consisting predominantly of anti-α-1.3 Gal that activates the complement system. Acute vascular rejection occurs because antibodies bind directly against α-1.3-Gal or other xenogeneic proteins.

1.2.2 Physiological compatibility

Besides the immunological rejection, the physiological compatibility is difficult to evaluate so far[52]. The survival time of transplanted organs is mostly too short to characterize its long-term functionality and the interaction with the transplant recipient in particular. In pig liver to NHP

xenotransplantations, livers from transgenic pigs for hCD55 or from GTKO pigs, which also expressed hCD46, the organ survival time ranged only between 7 to 9 days[53-55]. Recipients developed severe thrombocytopenia resulting in haemorrhage at various sites. In renal pig to NHP xenotransplantation from hCD55 transgenic pigs, the porcine kidneys mostly maintained plasma electrolyte homeostasis, hypoalbuminemia and an increased proteinuria as well as severe anemia. This is postulated to be due to molecular incompatibility of porcine erythropoietin with the primate Epo receptor. All these symptoms could be detected after transplantation and were described more in detail in Cowan *et al.*[56]. However, extensive *in vivo* and *ex vivo* data indicate that pig kidneys might function adequately in humans[57]. This was shown in for a kidney transplant from a pig with six modified genes which supported a baboon for 136 days[58]. For pig lung xenotransplantations the barriers seem to be greater than for other organs. This is demonstrated by the longest survival time of only 5 days of an NHP recipient. The pulmonary intravascular macrophages (PIMs), which can be additionally found to the resident pulmonary alveolar macrophages in pigs as well as the presence of metabolites like thromboxane and cytokines, might be one major aspect to explain the short survival time in lung xenotransplantations so far[59]. Nevertheless the genetic modifications performed in pigs have greatly lengthened the time that organs can survive in other animals. The heart of a α-gal transgenic pig, expressing hCD46 and hTM, was heterotropically transplanted into the abdomen of a baboon. It did not replace the baboon´s heart, but the baboon could survive for two and a half years with the transplant[60].

1.2.3 Microbiological risk in xenotransplantation

Another main hurdle is the microbiological risk associated with xenotransplantation. As with any form of transplantation, xenotransplantation carries the risk of the transmission of infectious pathogens with cells or tissue of the transplant. Additionally, there is the unique potential risk for the transmission of both known and unknown zoonotic agents into the human recipient, or even worse, the human population. However, the severity of risk is unknown in the absence of clinical trials[5].

In allotransplantation it is known that there is a risk of pathogen transmission from the donor to the recipient[61]. The most relevant pathogen focused on is the human cytomegalovirus (HCMV). This virus is transmitted to up to 92% of the transplant recipients and causes abdominal pain and diarrhoea, transplant rejection and opportunistic infections due to its immunosuppressive effect[62]. Moreover, the numbers of post-transplant proliferative disorder (PTLD) patients induced by Epstein Barr virus (EBV) are raising [63]. Besides HCMV and EBV other viruses could be transmitted to the recipient or already existing latent infections could get

reactivated through immunosuppressive therapy. Consequently, severe diseases, rejection of the transplant and the growth of tumours might occur. In addition, pathogens which are classified as non-pathogenic could become pathogenic because the physical barriers, like for example skin and mucosa, are circumvented and usually combined with a high immunosuppressive therapy. Most of the non-viral infectious microorganisms of pigs like *Trichinella spiralis*, *Strepptococcus suis*, *Toxoplasma gondii* or *Brucella suis* could be eliminated by spf-breeding, screening and selection of pathogen-free animals as well as with pathogen specific drug treatment.

The highest transmission risk that might occur is triggered off four groups of viruses[5]. There are first endogenous retroviruses which are, like in humans, part of the genome of all pigs and cannot be eradicated easily. Porcine endogenous retroviruses (PERVs) are transmitted vertically from generation to generation[64]. The second group is formed by herpesviruses which persist in the infected host and at the same time cannot be eliminated by the immune system. Thirdly, another risk could be induced by unknown potential infectious viruses which has already adapted to the host through coevolution, but do not cause a specific disease and cannot be detected by diagnostic methods so far. Moreover, viruses crossing the placenta (herpesviruses, circoviruses) which cannot be eliminated by spf-breeding are the fourth group to deal with regarding microbiological safety in xenotransplantation. For a longer period of time viruses were analysed when crossing the species-barrier from animals to humans and this has led to diseases characterized by high morbidity and mortality[65]. Around 60% of the roughly 400 emerging diseases since 1940 has stemmed from zoonoses[66].

Pigs may harbour several viruses with a pathogenic potential for humans or which might become dangerous for humans during xenotransplantation trials. This is, for example, known for Nipah viruses [67], Menangle virus[68], influenza [69], but also for hepatitis E virus (HEV)[70], porcine cytomegalovirus (PCMV)[71] and porcine lymphotropic herpesviruses -1, -2, -3 (PLHV)[72]. The outbreak of influenza H1N1 in 1918/1919 killed at least 40 million people and was analysed to originate from the swine influenza virus [73]. An overview of other known viruses which crossed the species barrier are shown in Table 2. Interestingly, all these zoonotic viruses where unknown in humans before they caused disease. For some of these viruses also human-to-human transmission was described (Marburg virus, Ebola virus, HIV)[74]. Several independent transmissions of SIV and the simian T-cell leukemia virus (STLV) led to the adaption of the virus from primates to humans giving rise to HIV and HTLV epidemic cases[75]. Phylogenetic analyses of HIV-1, HIV-2 and the human T-cell lymphotropic virus (HTLV) elucidated that their origins are simian lentiviruses[76-78] (Table 2).

virus	natural host	symptoms in natural host	symptoms in humans
Filovirus (Marburg, Ebola)	vervet monkey, chimpanzee, macaque	hemorrhagic fever, high mortality	hemorrhagic fever, high mortality (78%)
hanta virus	rodents (mouse, rat)	apathogenic	hemorrhagic fever, high mortality (50%)
hendra virus	bat	apathogenic	encephalitis
herpesvirus Saimiri	vervet monkey, baboon	apathogenic	meningoencephalitis
severe-acute-respiratory-syndrome-virus (SARS)	(maybe) bat	probably apathogenic	atypical lung infection
SIV	chimpanzee, mangabey, colobus, macaque	apathogenic	AIDS
STLV	baboon, macaque and others	T-cell leukemia	T-cell leukemia, immunodeficiency

Table 2 Transspecies transmission from animal to humans[74-75, 79-81]

1.3 Xenotransplantation in Germany

In Germany a consortium of basic and interdisciplinary scientists was established and funded by the Deutsche Forschungsgemeinschaft (DFG) Sonderforschungsbereich TRR 127. Immunologists, veterinarians and genetic engineers, physiologists, transplant surgeons and molecular biologists are on the way to develop pig-to-primate xenotransplantation to clinical application. The members of the consortium are doing research on the understanding of transplant rejection and on the regulation of the immunity of xenotransplants. The production of novel multitransgenic pigs can prevent endothelial cell activation, inflammation, thrombosis and immune rejection. The major focus lies on islet-cell transplantation and multi-transgenic pig to primate xenotransplantation of heart and kidneys in order to establish clinical application.

The microbiological safety plays a crucial role in xenotransplantation because severe obstacles could occur, ignoring the risk of pathogen transmission. The viruses listed in Table 3 are considered as potentially pathogenic and were characterized during this study. They will be therefore more explained in the next sections.

species	family	genus	genome/ Baltimore group	diseases in swine
PERV	*Retroviridae*	gammaretrovirus	ss (+)-RNA/ group VI	-
HEV	*Hepeviridae*	Calcivirus	ss (+)-RNA/ group VI	-
PCMV	*Herpesviridae*	*Betaherpesvirinae/ Roseolovirus*	ds-DNA	inclusion body rhinitis
PLHV-1,-2,-3	*Herpesviridae*	Gammaherpesvirinae	ds-DNA	PTLD-sysndrome
PCV2	*Circoviridae*	-	ss (-)-DNA/ group II	PMWS

Table 3 Potentially pathogenic viruses for human recipients in xenotransplantation

1.3.1 Porcine endogenous retrovirus

Porcine endogenous retroviruses (PERVs) are part of the family *Retroviridae.* With their unique lifecycle, their tumorigenic abilities and their role in acquired immune deficiency syndrome (AIDS), the group of retroviruses has attracted immense attention over the past 50 years[82-83]. Retroviruses are enveloped viruses with a single stranded (+)-RNA as genome. Their replication life cycle consists, compared to other types of viruses, of two unique viral enzymes, reverse transcriptase (RT) and integrase (IN)[84]. The RT permits the conversion of viral RNA

into DNA, followed by the integration of DNA with the IN forming a provirus in the host genome. Endogenous retroviruses (ERVs), like PERV, are transmitted vertically as a result of the infection of germ cells[85]. However, only under specific conditions can they be activated to produce exogenous, infectious particles[85].

As PERVs are part of the genome, they cannot be eliminated, contrary to other potentially zoonotic microorganisms in pigs by spf or dpf breeding[86]. Three groups of PERV are known that possess infectious potential. PERV-A and PERV-B are present in all pigs and are able to infect pig cells and human cells[64, 87-89]. PERV-C is common, but cannot be detected in every pig and only infects pig cells. A recombination between PERV-A and -C is possible and results in a highly replicating form named PERV-A/-C, which can also infect human primary cells *in vitro*[86]. Nevertheless, it could not be found integrated in the germ line.

So far, no transmission of the PERVs has been reported in several individuals having contact with pig tissues during either islet cell transplantation or *ex vivo* perfusion of porcine livers and spleens and no disease has been described for these viruses in pigs or humans until now[5]. PERVs could not be detected after pig to non-human primate transplantations with or without immunosuppressive therapy or the transplantation of encapsulated porcine islets in diabetic dogs[90-91]. Usually the survival time of organ recipients is too short to study the potential transmission of PERVs to the donor animal or no immunosuppressive therapy had been applied.

In addition, the choice of a suitable animal model to study PERV transmission is difficult. Non-human primates carry a mutated receptor for PERV-A and therefore the infection can be observed with reduced efficacy and is not productive[92-93]. Moreover, inoculation of high doses of PERV-A/-C in rhesus monkeys, baboons and pig tailed monkeys under strong immunosuppressive therapy failed to infect the animals[94]. Human cells carry a functional receptor, however, no transmission of PERVs was observed in the first clinical trial transplanting pig islet cells for the treatment of diabetes in New Zealand[95]. Nevertheless, it could be observed that infected human cells are able to transmit PERVs to unexposed human cells *in vitro* [96]. In order to prevent transmission of PERVs different efficient strategies have been developed during the last years:

I. Pigs with a low copy number and low expression on the RNA or protein level could be selected.
II. PERV-C free animals could be carefully chosen to avoid the recombination of PERV-A and PERV-C.
III. RNA-interference technology reduced the expression of PERV *in vitro*[97-99] and *in vivo*[100-101] successfully

IV. Gene editing using the zinc finger nuclease (ZFN) and the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated (CRISPR/Cas9) technology was performed. Since PERV is present in up to 100 copies per genome, it is a challenging task to knock out all proviruses. The use of ZFN induced toxic effects and destabilized the genome after cutting several sequences of PERV[102]. A breakthrough was achieved when 62 PERV proviruses could be disrupted in porcine kidney epithelial cell line (PK15) pig cells by CRISPR/Cas9 technology and demonstrated a >1000 fold reduction in PERV transmission to human cells[103].

1.3.2 Hepatitis E virus

Hepatitis E virus (HEV) is a non-enveloped virus with a single stranded (+)-RNA genome and part of the *Hepeviridae*. Four different genotypes of HEV are known, G1-G4: Genotypes 1 and 2 can only be found in humans and are endemic in large parts of Asia, Africa and Mexico. Genotypes 3 and 4 can be detected in humans and animals, like pigs, and are distributed in the industrial countries of Europe, as well as in Southeast Asia, North America and Australia[104]. Large epidemic outbreaks of GT1 and GT2 have been reported in developing countries, whereas in industrialized countries only sporadic HEV infections are reported[105]. In developed countries the source of infection cannot be identified. Because GT3 and GT4 are prevalent in pigs throughout the world and because pigs are the primary hosts for HEV, which does not cause a disease, it is suggested that the virus can be transmitted zoonotically by eating uncooked or undercooked pork products or meat from wild boar and deer[106]. Therefore only GT3 and GT4 might pose a risk in xenotransplantation using pig cells, tissue or organs.

The pathogenesis of HEV is not fully understood so far. One suspects that it is mainly transmitted enterically and that after oral ingestion of the virus, primary replication takes place in the intestinal tract and is then transported via the portal vein to the liver[107]. In humans infections can induce mild to moderate, self-limiting hepatitis but in pregnant women infections with GT1 and GT2 can lead to the loss of the foetus and death of the mother, with a mortality rate of approximately 30%. This is due to complications like eclampsia and haemorrhage in combination with or without acute liver failure[108].

HEV infection in pigs occurs at the age of 2-3 months and 80-100% of the pigs on commercial farms in the USA were infected. Moreover, it has been observed that swine veterinarians and pig traders or butchers are at a higher risk to be infected with HEV than other people in seroepidemiological studies[109]. Today HEV is characterized as a zoonotic virus infecting pigs but a wider range of other animals is also exposed to this risk[110]. Until now it has been difficult to evaluate the threat of HEV transmission during xenotransplantation. Although the infection

of GT3 and GT4 is usually harmless for healthy people with a stable immune system, in xenotransplant recipients under immunosuppressive therapy the risk of a xenozoonoses is even higher and might cause severe obstacles. Possible donor animals for xenotransplantation studies should be screened and tested negative. The fact that the virus is heterogenous causes problems to detect all subtypes. Nucleotide sequence variants make it difficult to design efficient, highly sensitive PCR-methods[111]. So far, the design of molecular tools to detect HEV RNA is based on known HEV sequences and might not be able to detect distant related forms[111], which makes it even harder to select HEV-free pigs for xenotransplantation. In addition, the virus load seems to be very low. Therefore the negative result of a tested animal leaves the question open whether the detection method was sensitive enough or whether it has been eliminated by the immune system. Other methods like serological testing via Western Blot or ELISA might be a solution to overcome this problem. The best material to detect HEV would be in liver samples or blood, but HEV can also spread in the gastrointestinal tract and can be detected in stool or faeces for several weeks after infections[112-113].

Two well-characterized non-transgenic pig breeds which have been discussed to be putative donor animals for xenotransplantation, have been screened for HEV: first the Auckland island pigs in New Zealand and second the Göttingen minipigs from Denmark. The islet cells of Auckland island pigs have already been used in the several clinical trials for encapsulated cell transplants[95, 114]. Although HEV is widely distributed in pigs all over New Zealand, the Auckland island pigs have been found negative for HEV[115]. Moreover, in a preclinical prospective pig to non-human primate trial and in all other clinical trials using islet cells from these animals, neither PERV nor other microorganisms including HEV could be detected in the recipients at several time points up to one year after transplantations[95, 116]. The Göttingen minipigs from Ellegaard in Denmark are a well-studied pig breed concerning their health status and genetics and are used worldwide for several biomedical investigations[117-118]. Göttingen minipigs are screened periodically for 27 bacteria, 16 viruses, three fungal organisms and four parasites but HEV was not on the distributor´s list to be screened for[119], HEV RNA as well as antibodies could be found in a low number of piglets and their mothers, when Göttingen minipigs were screened by real-time PCR or Western Blot[120]. For xenotransplantation it would be indispensable to choose HEV negative animals and select or eliminate the virus from putative donor herds. Moreover, an HEV infection can be effectively treated with ribavirin[121-122] which has been proved successful with allotransplant recipients infected with HEV GT3 or infected hematopoietic stem cell recipients[123-125].

1.3.3 Porcine cytomegalovirus

Porcine cytomegalovirus (PCMV) belongs to the family *Herpesviridae* and the subfamily Betaherpesvirinae. So far it is not assigned to any genus, but recent studies of the viral polymerase and major capsid protein have indicated that it is genetically closer related to human herpesvirus 6 and 7 in the genus *Roseolovirus* than to HCMV[126-128]. PCMV is common in pig populations all over the world and induces latent infections in pigs. The prevalence in a herd is greater than 90% and even 98% in Europe, North America and Japan. It was originally designated “inclusion body rhinitis” based on the observation of inclusion bodies in cells of the nasal mucosa of pigs suffering from rhinitis (Done 1955). The disease caused by PCMV is usually self-limiting but it can cause foetal and piglet deaths, runting, rhinitis and pneumonia[129].

No serotypes of PCMV have been identified and no other animal reservoirs and arthropoda vectors have been reported. Natural infections can be only found in pigs. In the pig population the virus is transmitted horizontally via the orosanal route and is also thought to occur in utero and perinatal. Experimental PCMV infection of pregnant sows with or without prior immunity of the virus showed transplacental transmission of the virus, which makes it even harder to eliminate the virus by spf-breeding[130-131].

Usually the infection of PCMV occurs through nasal droplets[132] and it spreads systemically. The primary replication starts in the nasal mucosa or Harderian glands. Then the site of the secondary replication varies with age. In piglets or growing pigs it spreads to the kidney tubules, the epididymis and mucous glands of the oesophagus, whereas in the fetus or neonates the infection takes place in the capillary endothelium and lymphoid tissue[130, 133]. This observation is also important for the detection of the virus regarding its eradication in animals bred for xenotransplantation.

Herpesviruses are typical viruses which can be activated during immunosuppression, cause common infectious complications and therefore play an important role in transplantation studies. In allotransplantation the HCMV is routinely transmitted to the human recipient and associated with acute allotransplant rejection or dysfunction of the allotransplant[63, 134-135]. The clinical risk associated with HCMV reactivation is dependent on the dose and strain of the virus and also the immune status of the recipient. Nevertheless, prophylaxis with the antiviral drug valacyclovir showed a reduction of the transplant rejection in a renal transplantation study in a placebo-controlled trial[136].

Since it was thought that herpesviruses are not able to infect other species than their evolutionary host, the finding that HCMV has infected pig cells, has changed this attitude[137]. The question is whether the porcine CMV could also be able to infect human cells. However, PCMV is considered to be a putative zoonotic pathogen[138]. In previous studies it could be

shown that PCMV can infect human fibroblasts[139] and that in pig to non-human primate transplantation studies using PCMV-infected donor pigs, it could be detected in the animal recipients[140-141]. Moreover, it was observed that PCMV transmission reduced the survival time of the transplant of two kidney xenotransplantation trials up to nearly three times. This was shown during transplantations of organs from GalT-KO pigs either in baboons[142] or cynomolgus monkeys[143]. The kidney-xenotransplants of PCMV-negative animals survived 53 days on average, whereas the survival rate of xenotransplants from PCMV-infected pigs had a reduced mean transplant survival of 14.1 days[142].

The infection and prevalence of PCMV in putative donor animals has not been studied well. The Auckland island pigs from New Zealand, which have already been used for xenotransplantation-studies were negative for PCMV, although the distribution of the virus is high in New Zealand pigs[95]. Göttingen minipigs have not been screened for PCMV yet and there is also not much data about the prevalence in multitransgenic pigs especially bred for xenotransplantation. When adult large white pigs expressing CD55 were screened for PCMV, virus could be found in nearly every organ, although the titer was low[144].

Transmission of PCMV could be better avoided by the treatment with ganciclovir or cidofovir than by foscarnet and acyclovir[145]. Foscarnet and Acyclovir showed an inhibition to a lesser extent[145]. Furthermore, the transmission to swine offspring might be prevented by Caesarean delivery and strict isolation of donor animals in spf-facilities.

1.3.4 Porcine lymphotropic herpes viruses -1,-2 and -3

The detection of viral sequences in leukocytes and lymphoid organs of healthy pigs has led to the discovery of two of the first porcine herpesviruses belonging to the subfamily *Gammaherpesvirinae*: porcine lymphotropic herpesvirus 1 (PLHV-1) and porcine lymphotropic herpesvirus 2 (PLHV-2)[146]. In 2003 the porcine lymphotropic herpesvirus 3 (PLHV-3) was identified[147]. PLHVs are distributed in pig breeds all over the world and can be efficiently detected in spleen or lung samples. They have been found in domestic pigs from Germany, France, Italy, Belgium, Ireland, the United Kingdom, the USA, Australia and Vietnam[114, 146, 148-149]. So far little is known about their pathogenic potential in humans. But the presence of these herpesviruses in usually healthy pigs and the role of herpesviruses in transplantation studies has led to the question if they might be a risk during xenotransplantation trials. Different aspects like the worldwide prevalence of these viruses, the difficulties to eliminate them from potential donor animals[150] and the association with a PTLD-syndrome in immunosuppressed miniature swine have made them of special interest[151]. Moreover, oncogenic potential has been reported for gammaherpesviruses[152] . Cross-species transmission of gammaherpesviruses and increased pathogenicity has been observed for the related viruses

alcelaphine herpesvirus-1 (AlHV-1) and Ovine Herpesvirus 2 (OvHV-2)[153]. Their genomes are also related to those of human gammaherpesviruses like Kaposi sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), and EBV which are associated with non-Hodgkin lymphomas in immunocompromised hosts[146-147, 149, 154].

PLHVs have the potential to cause a lymphoproliferative disease in experimentally immunosuppressed pigs which is comparable to a PTLD in humans and is characterized by a high mortality rate. During a PTLD the patients might develop mononucleosis-like lesions or polyclonal polymorphic B-cell hyperplasia. The uncontrolled proliferation of B-lymphocytes may lead to the growth of tumours and affects nearly 10% of the patients in allotransplantation[155-156]. Moreover, it could be observed that after T-cell depleted allogeneic stem cell transplantation PLHV-1 was leading to death because B-cell lymphoproliferation occured[151]. Although no infection through PLHVs of human cell lines or humans has been reported so far, the risk of transmission to xenotransplant recipients could be promoted by the treatment of immunosuppressive drugs. Therefore the PLHV in the xenotransplant might recombine or coactivate with other human herpesviruses like HCMV, HHV-8 or EBV in the post-transplant period and might form a new even more pathogenic virus species[157]. The characterization of PLHVs is extremely limited by the lack of a working cell culture system. So far nothing is known about the prevalence and expression of PLHVs in Göttingen minipigs, as well as Aachen minipigs and pigs especially bred for xenotransplantation.

However, in order to avoid the risk during xenotransplantation, the breeding of PLHV-free pigs for xenotransplantation is indispensable. This might be a hard condition because early weaning of the piglets has not excluded PLHV. Nevertheless, promising results were obtained by Caesarean delivery and scrupulous barrier reared breeding conditions indicating that PLHVs might be transmitted horizontally but not vertically.

1.3.5 Porcine circoviruses

The porcine circovirus is a non-enveloped single stranded DNA virus and a member of the family *Circoviridae*. In the beginning there was no correlation found between a specific disease and the virus and therefore it had been considered to be non-pathogenic. This is true for the porcine circovirus 1 (PCV1), but after a couple of years the porcine circovirus 2 (PCV2) was found to be associated with several different diseases, especially postweaning multisystemic wasting syndrome (PMWS) in swine. Pigs with PMWS show different clinical symptoms like debility, diarrhea, dyspnea, palpable lymphadenopathy, pallor or icterus finally inducing immune suppression depending on the viral subtype, genetic factors of the host and co-infections[158]. The lesions caused by PMWS could be reproduced experimentally after inoculation of PCV2 cell culture isolates in healthy piglets. Nevertheless, the full expression of

PMWS could not be observed, indicating that the presence of other viruses such as porcine parvovirus or porcine reproductive and respiratory syndrome (PRRS) virus might be required[159]. Pigs suffering from PMWS either die within 6-8 days or remain in growth and become a runt, which leads to considerable financial losses for the animal owner.

PCVs are quite stable in the pig environment and hard to eliminate and they are resistant under high temperatures and pH conditions[160]. The presence of PCV-1 can be detected throughout the world because antibodies were found in Germany[161], Canada[162], New Zealand[163], Northern Ireland[159] and Great Britain[164]. The prevalence of PCV2 was analysed in wild boars and breeding herds but numbers in the literature ranged from 1% to 100% infection rate[165-168].

In xenotransplantation there is obviously only a risk associated with PCV2. Healthy pigs for xenotransplantation are a prerequisite leading to higher quality and lifetime for the transplant. PCVs are transmitted horizontally and infect cells of the immune system[169]. Therefore the risk of the transmission of the virus to the human recipient by the infection of human epithelial and endothelial cells or macrophages might be possible[170]. Moreover, the fact that PCV induces immunosuppression, makes it easier for other zoonotic pathogens to infect the transplant recipient and viruses which are suppressed and controlled by the immune system of the host can also be reactivated. Nevertheless, the question whether direct infection of PCV2 is possible to the human recipient has not been solved yet. The infection of human cell lines with PCV2 lead to cytopathogenic effects, however, vaccination with the live-attenuated rotavirus vaccine Rotarix® (GlaxoSmithKline, Rixenxart, Belgium) contaminated with PCV1 and PCV2 particles has not caused an infection of the vaccinated patients[171-172].

Pigs used for xenotransplantation should be vaccinated before housing them in spf-facilities. Several vaccines are commercially available to protect pigs from PCV2, for example Porcilis® PCV (MSD Animal Health, Boxmeer, The Netherlands) for protection of pigs from three weeks and older, Ingelvac CicroFlex® (Boehringer Ingelheim, Ingelheim, Germany) for use in pigs of two weeks and older, Circovac® (Merial, Lyon, France) for use in pigs three weeks and older, often leading to hyperthermia and other systemic reactions[173].

2 Aims of the study

Xenotransplantation provides a potentially promising solution to overcome the shortage of human organs and tissues. However, the potential to introduce new infections from xenotransplants of donor animals into the human population has been of major concern. Some of these pathogens lead to immunosuppression, transplant rejection or might be zoonotic and diseases can be transmitted from animals to humans. Consequently, in many countries considerable effort has been made to improve safety and to develop guidelines for the husbandry of donor animals and the monitoring of transplant recipients.

Against this backround, the aim of the study was to analyse the prevalence and infection of different porcine viruses in putative donor animals in order to obtain safe and healthy donor pigs for xenotransplantation studies. Porcine endogenous retroviruses (PERVs), porcine cytomegalovirus (PCMV), porcine lymphotropic herpesviruses (PLHVs), porcine circovirus 2 (PCV2) and hepatitis E virus (HEV) are of special interest because of the characteristics listed above. As a prerequisite, highly sensitive and specific detection methods should be developed. PCR-based methods, real-time PCR and real-time RT-PCR specific for all the viruses listed above, as well as immunological methods, like Western blot analysis, were available for some viruses. For others new primers/probes or antigens to detect antibody reactions had to be developed and optimized.

Moreover, these methods should be used to investigate the safety and transmission in preclinical xenotransplantation trials, like islet cell transplantation or heart transplantation in non-human primate studies.

The generated data should help to assess the risk during xenotransplantation, to monitor and select healthy donor pigs as well as suitable recipient animals. Moreover, these detection methods are important for effective elimination programs based on treatment, vaccination, caesarean delivery, early weaning and embryo transfer to prevent their transmission. The data and results were published in 7 publications.

3 Cumulative part

List of publications

publication I	Plotzki E, Wolf-van Buerck L, Knauf Y, Becker T, Maetz-Rensing K, Schuster M, Baehr A, Klymiuk N, Wolf E, Seissler J, Denner J.: *Virus safety of islet cell transplantation from transgenic pigs to marmosets*. Virus Res. 2015 Jun 2;204:95-102. **Own contribution: A= ~70% B= ~30%**
publication II	Abicht JM, Mayr TA, Reichart B, Plotzki E, Güthoff S, Falkenau A, Kind A, Denner J.: *Hepatic Failure After Pig Heart Transplantation Into a Baboon: No Involvement of Porcine Hepatitis E Virus.* Ann Transplant. 2016 Jan 7; 21:12-6. **Own contribution: A= ~20% B= ~20%**
publication III	Plotzki E, Keller M, Ehlers B, Denner J.: *Immunological methods for the detection of porcine lymphotropic herpesviruses (PLHV)*.J Virol Methods. 2016 Jul; 233:72-7. **Own contribution: A= ~50% B= ~40%**
publication IV	Plotzki E, Heinrichs G, Kubícková B, Ulrich RG, Denner J.: *Microbiological characterization of a newly established pig breed, Aachen Minipigs.* Xenotransplantation. 2016 Mar;23(2):159-67. **Own contribution: A= ~70% B= ~30%**
publication V	Plotzki E, Keller M, Ivanusic D, Denner J.: *A new Western blot assay for the detection of porcine cytomegalovirus (PCMV).* J Immunol Methods. 2016 Oct; 437:37-42. **Own contribution: A= ~50% B= ~40%**
publication VI	Morozov VA, Plotzki E, Rotem A, Barkai U, Denner J.: *Extended microbiological characterization of Göttingen minipigs: porcine cytomegalovirus and other viruses.* Xenotransplantation. 2016 Nov; 23(6):490-496. **Own contribution: A= ~40% B= ~20%**
publication VII	Heinze J, Plotzki E, Denner J.: *Virus safety of Xenotransplantation: Prevalence of porcine Circovirus (PCV2) in pigs.* Ann of Virol and Research. 2016 Dec **Own contribution: A= ~40% B= ~30%**

A= own contribution concerning design, implementation, analysing of data and creation of figures and tables

B= own contribution in writing the manuscript

The following chapter contains 7 publications, which were published as research articles in international journals undergoing a professional peer review. These publications altogether focus on the virological safety in xenotransplantation and build the main part of this thesis. They are either publications concerning method development (publications III, IV, VI), screening and finding of safe and healthy donor pigs for xenotransplantation trials (publications IV, VI, VII) or screening of donor and recipient animals in preclinical trials, for example an islet cell transplantation from transgenic pigs to marmosets as well as a heart transplantation from a multitransgenic pig to a baboon (publications I, II).

3.1 Publication I: Virus safety of islet cell transplantation from transgenic pigs to marmosets

Link:

https://doi.org/10.1016/j.virusres.2015.04.016

Quote:

Plotzki E, Wolf-van Buerck L, Knauf Y, Becker T, Maetz-Rensing K, Schuster M, Baehr A, Klymiuk N, Wolf E, Seissler J, Denner J.:
Virus safety of islet cell transplantation from transgenic pigs to marmosets.
Virus Res. 2015 Jun 2;204:95-102.

3.2 Publication II: Hepatic Failure After Pig Heart Transplantation Into a Baboon: No Involvement of Porcine Hepatitis E Virus

Link:

https://doi.org/10.12659/AOT.896544

https://www.annalsoftransplantation.com/download/index/idArt/896544

Quote:
Abicht JM, Mayr TA, Reichart B, Plotzki E, Güthoff S, Falkenau A, Kind A, Denner J.:
Hepatic Failure After Pig Heart Transplantation Into a Baboon: No Involvement of Porcine Hepatitis E Virus.
Ann Transplant. 2016 Jan 7; 21:12-6.

3.3 Publication III: Immunological methods for the detection of porcine lymphotropic herpesviruses (PLHV)

Link:

https://doi.org/10.1016/j.jviromet.2016.02.017

Quote:

Plotzki E, Keller M, Ehlers B, Denner J.:
Immunological methods for the detection of porcine lymphotropic herpesviruses (PLHV).
J Virol Methods. 2016 Jul; 233:72-7.

3.4 Publication IV: Microbiological characterization of a newly established pig breed, Aachen minipigs

Link:

https://doi.org/10.1111/xen.12233

Quote:

Plotzki E, Heinrichs G, Kubícková B, Ulrich RG, Denner J.:
Microbiological characterization of a newly established pig breed, Aachen Minipigs. Xenotransplantation. 2016 Mar;23(2):159-67.

3.5 Publication V: A new Western blot assay for the detection of porcine cytomegalovirus (PCMV)

Link:

https://doi.org/10.1016/j.jim.2016.08.001

Quote:

Plotzki E, Keller M, Ivanusic D, Denner J.:
A new Western blot assay for the detection of porcine cytomegalovirus (PCMV).
J Immunol Methods. 2016 Oct; 437:37-42.

3.6 Publication VI: Extended microbiological characterization of Göttingen minipigs: porcine cytomegalovirus and other viruses

Link:

https://doi.org/10.1111/xen.12265

Quote:

Morozov VA, Plotzki E, Rotem A, Barkai U, Denner J.:
Extended microbiological characterization of Göttingen minipigs: porcine cytomegalovirus and other viruses.
Xenotransplantation. 2016 Nov; 23(6):490-496.

3.7 Publication VII: Virus Safety of Xenotransplantation: Prevalence of Porcine Circovirus 2 (PCV2) in Pigs

Link:

http://dx.doi.org/10.25646/2426

Quote:

Heinze J, Plotzki E, Denner J.:
Virus safety of Xenotransplantation: Prevalence of porcine Circovirus (PCV2) in pigs.
Ann of Virol and Research. 2016 Dec

4 Discussion

The generated data help to assess the risks during xenotransplantation, a promising solution to overcome the shortage of human organs and tissues. There is a potential risk that new infections from xenotransplants of donor animals could be introduced into the human population. The transmitted pathogens may lead to immunosuppression, transplant rejection or might be zoonotic. Therefore the monitoring and selection of healthy donor pigs is indispensable as well as the choice of suitable recipient animals.

In the present publications, different data regarding the microbiological safety in xenotransplantation have been collected and aspects were illuminated.
Publications III, IV, VI focus on method development. Especially highly sensitive PCR and immunological methods for the porcine herpesviruses had to be established, because none of these methods have been used in our group at RKI before. These methods will as well as their advantages and disadvantages be discussed. The screening and finding of safe and healthy donor pigs for xenotransplantation trials were analysed mainly in publications IV, VI, VII, investigating the health status of (I) Göttingen minipigs, housing under spf- conditions in Ellegaard, Denmark, (II) Aachen minipigs, a new pig breed in western Germany, (III) multitransgenic pigs, especially bred for xenotransplantation and (IV) slaughterhouse pigs from an abattoir close to Berlin. The finding of suitable donor animals will be discussed here. Moreover, the screening of donor and recipient animals in preclinical trials, like an islet cell transplantation from transgenic pigs to marmosets as well as a heart transplantation from a multitransgenic pig to a baboon, was examined in publications I and II.

4.1 Method development

4.1.1 PCR-based methods

The selection of safe and healthy donor animals for xenotransplantation trials is a major goal. Acute viral infection can be diagnosed by clinical manifestations, though some viral infections might be symptomless and chronical. For this purpose, highly sensitive detection methods are required to allow a precise selection preventing potential transmission of infectious microorganisms to the xenotransplant recipient. At the beginning of the project conventional PCR methods have already been available for some of the analysed viruses like PERV, PLHVs and PCV2. Nevertheless, real-time PCR has been proven to be similarly sensitive, easier to optimize and, moreover, it provides the opportunity of absolute quantification of viral copies by

the use of a reference plasmid and the generation of calibration curves[174]. Consequently, for all methods used, the detection limit could be estimated and its own reference plasmid was cloned. Already published primers were compared with new primer pairs. Real-time PCRs were compared with conventional PCRs. The detection limit calculated of every PCR actually used for the characterization studies is shown in Table 4.

Virus	Detection limit (copies per genome)	Reference
PERV-A/ -B	$1x10^1$ (own quantification)	[175]
PERV-C	$1x10^2$	[176]
HEV	$1x10^1$	[120, 177]
PCMV	$2x10^1$	[141]
PLHV -1/ -2	$1x10^2$ (own quantification)	Publication IV
PLHV-3	$1x10^2$	[147]
PCV2	$1x10^3$	[178]

Table 4 Detection limit of PCR-based methods used for screening of putative donor and recipient animals

During the optimization of these PCR methods different parameters like annealing temperature, elongation time, primer and probe concentration, DMSO concentration and $MgCl_2$ concentration were changed (Publication VI). Another very important parameter is the type and quality of the biological material in order to detect the different viruses in putative donor pigs. Blood and sera are the most frequently used materials in commercial farms and facilities, but this is also a stressful procedure, especially for piglets[179]. Moreover, the detection of viruses like HEV, PCMV and PLHVs is even more successful when using organs or ear biopsies because of the different expression in different organs. Parameters like the perfect material, time points of detection and isolation methods for RNA or DNA show an effect on the detection of a virus.

The expression of PERV is higher in lung, liver, spleen and lymph node than in other organs. The copy number is increased in adult pigs compared to newborns[175, 180].

For the detection of HEV blood, sera and faeces are the materials of choice[181]. However, liver samples proved to be most effective for the detection of HEV. This could be explained by the quality of RNA and the higher amount obtained from the extraction of organs.

PCMV infection was found to be easily detectable in organs like spleen, liver, heart, lung and lymph node, but the detection and quantification in blood was problematic in some cases. This is why PCMV infection in adult pigs is latent and when only using blood as material, the virus is difficult to detect. In order to overcome this problem, the isolation and cultivation of peripheral

blood mononuclear cells (PBMCs) showed effective results. After cultivating PBMCs for 5 days, PCMV could be detected in samples which have been found negative in blood samples (Fiebig, personal communication). In previous studies the same result was achieved. No PCMV was detected in blood, faeces and urine, nasal and oral swabs, but it was found in a low copy number in spleens and PBMCs[129].

For PLHVs best detection results were achieved in spleen and liver samples. It was only possible to detect PLHVs in blood samples of slaughterhouse pigs and Aachen minipigs. This might be due to a higher copy number and the fact that the animals were not kept under spf-conditions.

PCV2 could be detected in blood and liver samples, but only in a low number of animals of Göttingen minipigs and Aachen minipigs. This might be explained due to their vaccination. PERV-A and -B can be detected in every organ, but highest expression levels could be found in kidney, lung and spleen[175, 182]. The copy number of PERV not only differs in organs but also in different pig breeds and in different animals of the same pig breeds[183]. This observation could also be found in a comparison of large pigs and minipig breeds in China[184]. Normal size pig breeds harboured 179 copy number variations, whereas two Chinese minipig breeds only showed 21 copy number variants. 60 copy number variants could be detected for three western pig breeds[184].

Only multitransgenic pigs without PERV-C were analysed. The copy numbers of PERV and therefore the evidence for replication and de novo integration are poorly understood so far[85]. In contrast to HERV for example, where the copy number is identical in every cell of one individual, PERVs are still replicating. This can concluded by the recombination of human tropic PERV-A and pig tropic PERV-C. Moreover, the recombination PERV-A/-C cannot be found in the germ line, only in the cellular DNA from pig organs[185]. Regarding the analysed pig breeds it could be a possible explanation that the replication frequency in some pigs is higher than in others and that there are usually not many different pigs used for breeding procedures. That is why the copy number in animals of the same cohort is very similar. For xenotransplantation studies it would be optimal to choose pigs without PERV-C and with a low copy number of PERV. Attempts to eradicate PERVs with gene editing methods like Tal effector nucleases (TALEN)[186], zinc finger nuclease (ZFN)[102] and CRISPR/Cas9 have shown, especially in the case of CRISPR/Cas9, promising results, because all 62 copies were disrupted in PK15 cells[96, 103]. An important breakthrough was achieved when the first 37 PERV-inactivated piglets were recently generated using the CRISPR/Cas9-method[96]. New methods like digital droplet PCR (ddPCR) and higher coverage sequencing (deep sequencing)[187] will be one option to determine exact copy numbers of proviruses in pigs used for xenotransplantation trials in future and to regularly check-up generated PERV-inactivated pigs .

In order to examine the PCR results and have a look at the antibody response against the viruses of interest, antigens for PCMV and PLHVs were cloned, expressed and purified. A Western Blot for PERVs with recombinant proteins and viral lysate has already been developed in the group before [188-190]. The recombinant GT3 ORF 2-HEV antigen[191] was kindly provided by PD Dr. R. G. Ulrich from the Friedrich-Loeffler-Institute in Riems.

PCR methods are used to detect the genome and therefore the subtype of the different viruses. Moreover, the determination of the viral load is possible with a standard and calibration curve and statements can be made about the infectivity of the patient or animal[192]. Results obtained from antibody-assays like Western Blot or ELISA are used to analyse if there has already been an infection with the virus in the past. If no antibodies can be found either there has been no contact or it is at a very early stage of infection. Testing of IgM (early stage of infection) or IgG (late stage of infection) can help to appoint the time in the infection cycle.

4.2 Prevalence in tested pig breeds

The investigations performed in our studies show that every analysed virus could be found in the tested animal breeds (table 5). Thus it will be a difficult task to eliminate putative infectious viruses from the herds.

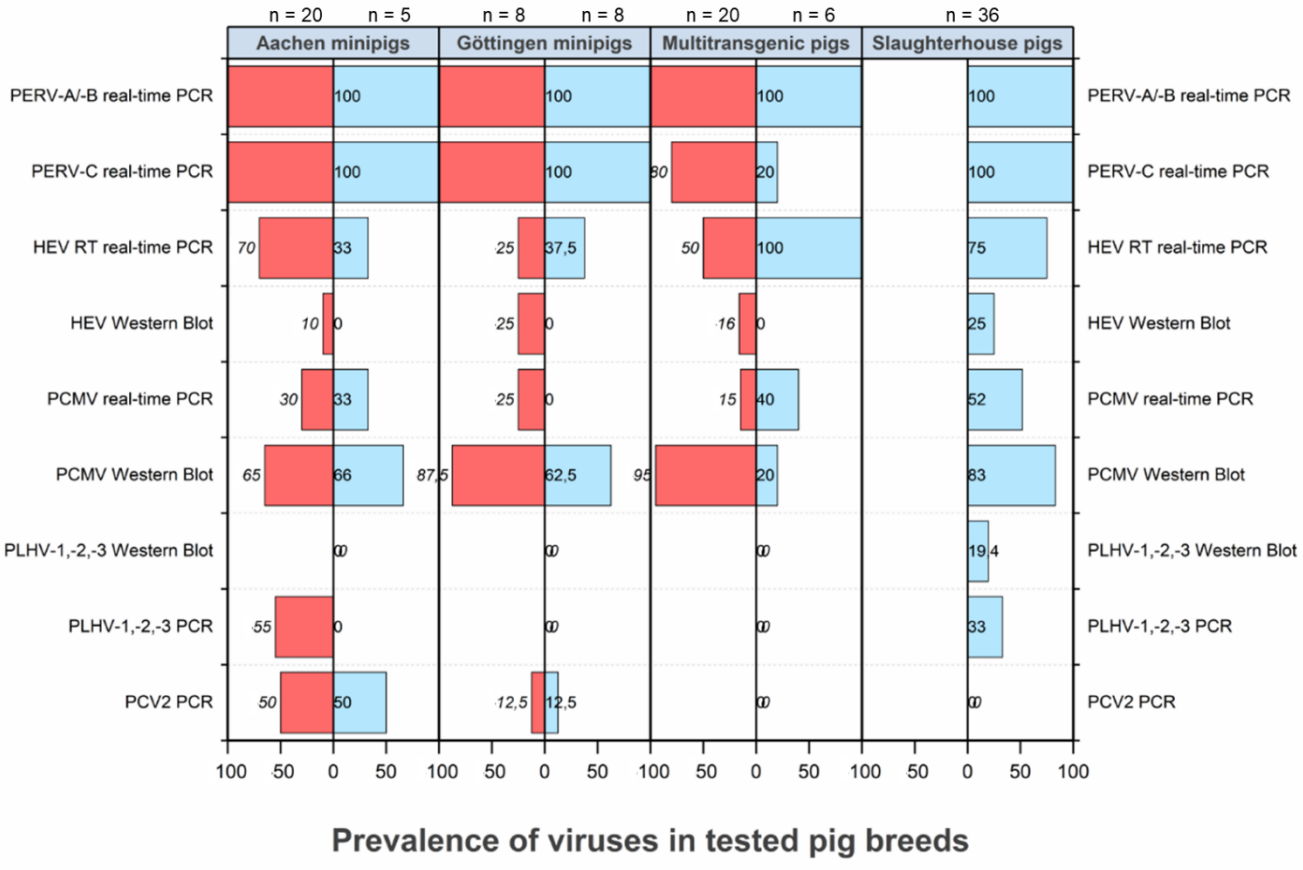

Figure 3 Prevalence of viruses in tested pig breeds
The prevalence of the viruses is shown in the tested pig breeds. It is subdivided in female (red) and male (blue) pigs. The prevalence is given as percentages. 0 indicates that all tested pigs were negative for the specific virus. n represents the total number of pigs. For the slaughterhouse pigs only the percentages for males are shown, because there is no information about sex of the pigs.

All pigs were positive for PERV-A and -B. Interestingly 5 out of 25 multitransgenic pigs were free of PERV-C, which is important regarding the fact that they are not able to form a highly replicating recombination subtype PERV-A/C[185]. This is in concordance with literature, because only a low number of animals could be found negative for PERV-C[176]. So far a Chinese group found a negative group of 37 animals[193]. These animals should be analysed more closely. However, the negative results might be due to the detection limit of the used PCR-method or the quality of the tested material. The detection rate of HEV differed between 31% (total) in Göttingen minipigs and 75% (total) in slaughterhouse pigs. This might also be due to the fact that Göttingen minipigs live under spf-conditions. Slaughterhouse pigs and Aachen minipigs, living under normal housing conditions, showed the highest prevalence. Moreover, most studies detected a higher prevalence of viral HEV-RNA in faeces of pigs

younger than 4 months of age than in older pigs[194]. This could explain the higher prevalence of HEV in multitransgenic pigs (2 weeks to 9 months) and Aachen minipigs (3 to 12 months), because younger animals were tested in comparison to older Göttingen minipigs used for testing (3 to 18 months). A study in Europe revealed that the HEV prevalence in weaners ranged from 8% to 13%, from 20% to 40% in growers and from 8% to 73% in fattening pigs and pigs at slaughter[195]. This is in agreement with the prevalence in our slaughterhouse pigs. But the seroprevalence of anti-HEV IgG antibodies was lower than expected. Studies showed that seroprevalence in Italy is very high and can reach over 90% in pigs around one year of age[196]. The differences in seroprevalence could be explained by the use of different antigens. In the study described, Di Bartolo *et al.* used a commercial ELISA BioChain kit, whereas in our studies a novel sensitive and specific antigen with the carboxy-terminal segment of HEV-GT3 was used in a Western Blot[191]. Additionally, the number of analysed sows and piglets was low and the detection rates obtained might not be representative for all pig populations in Italy.

Compared with HEV the prevalence of PCMV detected by PCR is lower. That is why PCMV is frequent, but often latent[197]. The seroprevalence of PCMV is high matching with results of our study. In a province in China, for example, the seroprevalence ranges from 94% to 98%[198].

PLHVs could not be detected in Göttingen minipigs kept under spf-conditions and only 1 out of 25 multitransgenic pigs was found positive (6%). 11 out of 26 Aachen minipigs were positive for PLHVs (42%) and 12 out of 36 slaughterhouse pigs (33%). Only 7 slaughterhouse pigs showed an antibody response against PLHVs (19.4%). This result might be due to the fact that pigs did not induce an antibody production. This observation should be further proved by the use of alternative methods using other immunodominant antigens or immunofluorescence studies. In other studies prevalences around 48% to 62% for PLHV-1, 16%-41% for PLHV-2 and 54-78% for PLHV-3 where estimated in German domestic pigs[133]. Moreover, when Brema *et al.*[199] used the same sequence for their recombinant glycoprotein B, the seropositivity ranged from 38%-100% positivity[199]. The positive results for PCV2 in Göttingen minipigs (18.75% in total) and Aachen minipigs (50% in total) might be due to the lack of vaccination, and because organ samples like liver and kidney were available. Only blood samples and no organs were tested from slaughterhouse pigs. The genetically modified pigs especially produced for xenotransplantation studies had been vaccinated before testing.

Summarizing the results of finding safe and healthy donor pigs is a demanding task that needs periodical strict and time consuming testing in future. The detection of the viruses is challenging, especially when only using blood and sera, because no organ samples are available in living animals. The viral load and amount of DNA and RNA will be less in blood and sera than in organ samples. Nevertheless, some of the multitransgenic pigs, negative for PERV-C, were also negative for HEV, PCMV, PLHVs and PCV2. These animals should be selected, separated from the herd to avoid new infections and handled under highly hygienic

conditions. Other putative donor animals could be treated with drugs like ribavirin (HEV) and vaccination for PCV2. Transplant recipients might be medicated with antiviral prophylaxis like mTor-inhibitors for PCMV, such as everolismus[200-201]. Additionally, it might be an option to import and use pigs which have already been used for clinical islet-cell transplantation trials before, like the Auckland island pigs[95, 202]. 15 Auckland island pigs were selected and analysed in our lab using the established detection methods. None of these pigs were found positive for HEV and PCV2. 6 of these animals were negative for PERV-C and results for PCMV in PCR showed controversial results assuming that the viral load is very low in some of these pigs. However, no antibodies of HEV and PCMV could be detected in the sera indicating that this pig breed seems well-suited for xenotransplantation studies.

During the first clinical trial of an islet cell transplantation from multitransgenic pigs in marmosets no transmission of either PERV, HEV, PCMV, PLHV and PCV2 could be observed after 6 months. The data obtained are comparable with data from previous clinical xenotransplantations. When blood and sera were tested of burn patients who had received living pig-skin dressings, no evidence for PERV transmission was observed in up to 34 years. Moreover, from October 2009 to March 2011, the company Living Cell Technologies (LCT) performed a clinical trial in New Zealand with 14 patients[203]. Their clinical product, called DIABECELL®, is currently in late stage clinical trials and islet cells of Auckland island pigs had been used[204]. The donor animals had been carefully screened for different potentially zoonotic microorganisms[116, 205] and no transmission was observed[95]. Patients implanted with the cells did not show any evidence for immune rejection or infection and survived more than nine years[204]. Even when immunosuppression was applied, no transmission of PERV was detected when extracorporeal kidney, spleen and liver perfusion-experiments were performed[206].
Moreover no transmission of HEV could be detected in any preclinical and first clinical xenotransplantation trials using Auckland island pigs [95, 116, 202]. This was also shown in triple-transgenic pig-to-baboon heart transplantation (publication II). Nevertheless, the zoonotic potential of HEV is known[207] and human infections are rising in Germany[208]. During the same pig-to-baboon heart transplantation the transmission of PCMV was analysed[209]. PCMV could be detected in the blood of a immunosuppressed recipient baboon after 29 days, but it could not be detected in the blood and serum of the donor animal[209]. Another transmission of PCMV was observed during the heart transplantation of another baboon after 40 days (Fiebig *et al*., not published). PCMV could be detected at a high level in the spleen and at lower amounts in blood and liver of the recipient-baboon. These findings are the first observations that PCMV was transmitted during xenotransplantation trials. Similar results were obtained after a baboon-to-human liver xenotransplantation. On day 29, 36 and 42 replication-competent baboon CMV (BaCMV) could be isolated from blood samples of the human recipient and showed a potential

zoonotic transmission from the source animal to humans during a xenotransplantation trial[210]. Moreover, the treatment with immunosuppressive drugs rises the risk of reactivation of latent infections by BaCMV and PCMV. In thymokidney xenotransplantation trials increased BaCMV copy numbers and an upregulation of PCMV was found. Additionally, infection of PCMV was associated with necrosis in one xenotransplant[140].

To avoid future transmissions of porcine viruses, more sensitive detection methods, different time points of testing and different source materials, including oral and anal swabs, should be used. The newly developed detection methods are a prerequisite for the selection of virus-free pigs for transplantation trials as well as elimination programs based on treatment, vaccination, Caesarean delivery, early weaning and embryo transfer.

5 Conclusion and Outlook

Xenotransplantation is a promising strategy to overcome the shortage of human donor organs. Nevertheless, there is a potential to introduce new infections from xenotransplants of pig donor animals into the human population. To assess the risk during xenotransplantation and to monitor and select healthy donor pigs as well as suitable recipient animals using highly sensitive and specific detection methods was the goal of the study. During the study PCR-based methods, real-time PCR and real-time RT-PCR specific for putative zoonotic viruses, as well as immunological methods like Western blot analysis were developed and optimized. These methods should be used to investigate the safety and transmission in potential donor animals and recipient animals after preclinical xenotransplantation trials like islet cell transplantation or heart transplantation in non-human primate studies.

To summarise,

1. The focus of publication I was the virological safety during a transplantation of pig islet cells from wildtype and transgenic pigs for the treatment of diabetes. This might be a more effective approach compared with the application of insulin. The prevalence of PERVs, which are present in the genome of all pigs and which may infect human cells, as well as of porcine herpesviruses in donor pigs and their potential transmission to non-human primate recipients was investigated using PCR-based methods and immunological methods. Despite the fact that all three subtypes of PERV were present in all pigs and PCMV was found in some of the pigs, neither PERVs nor PCMV were found in the recipient animals. Porcine lymphotropic herpes viruses were also not found in the donor pigs. In order to study whether a transmission of PERV to the recipient animals marmoset (*C. jacchus*) is possible, the PERV-receptor of the marmosets was sequenced and analysed. Nevertheless, no mutation in position 42 was investigated, which is the main reason for poor replication.

2. In publication II an increase of liver parameters was observed in one baboon after transplantation of a pig hearts into baboons. In order to evaluate whether HEV was involved in the pathological changes, the donor pig and the recipient baboon were screened for the presence of HEV. Screening for HEV was performed using highly sensitive and specific PCR methods as well as immunological screening for HEV-specific antibodies. However, HEV was not detected in the donor pig or the baboon recipient 57 after 29 days. At necropsy, histopathological examination of liver sections showed acute coagulative necrosis of hepatocytes and haemorrhage, but minimal inflammatory cell activity.

Therefore, it can be assumed that the liver failure in the recipient animal was not due to the transmission of porcine HEV. It could have been caused by the onset of cardiac failure related to delayed transplant rejection.

3. The focus in publication III lies on the immunological method development for porcine PLHV-1, -2, and -3. Since PLHVs may be transmitted from donor pigs to the human transplant recipient when xenotransplantation using pig cells, tissues or organs will be performed, sensitive and specific methods should be developed to detect and eliminate PLHVs. Here we describe an ELISA and a Western blot assay using recombinant glycoprotein B of PLHV-1 and a positive goat serum obtained by immunisation. Using both assays, we analysed the presence of specific antibodies in different pig breeds as well as in workers in German slaughterhouses. In some animals antibodies were found, but not in human individuals.

4. In publication IV the characterization of a new minipig breed, which could be used as putative donor animal for xenotransplantation trials in future, was analysed. The Aachen Minipig (AaMP) is a pig breed recently established close to the town Aachen in Germany. A selection of animals was tested for the prevalence and expression of PERVs and the presence of some selected microorganisms, among them HEV, PCMV, and PLHVs using highly sensitive and specific PCR and RT-PCR methods. In addition, the antibody-reaction against HEV and PLHV were analysed. PERV-A, PERV-B, and PERV-C sequences were found in the genome of all Aachen Minipigs (AaMP). HEV RNA was found by real-time RTPCR in most, and DNA of PCMV, PLHV-2, and PLHV-3 was found by PCR in some animals. The animals were free of eight other microorganisms tested, but some were seropositive for porcine circovirus 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine epidemic diarrhea virus (PEDV). Based on medical examinations by veterinarians, the AaMP are in good health and seem to harbour only few microorganisms. For use as donor pigs in xenotransplantation, their health status should be further improved by the elimination of the detected viruses and by selection of negative animals, Caesarean section, and vaccination.

5. The design of a Western blot assay using recombinant proteins corresponding to two domains of the glycoprotein B of PCMV was described for the first time in publication V. With this assay, the presence of PCMV-specific antibodies in different pig breeds was analysed. Antibodies were detected in a high number of animals (up to 83 % in certain breeds). The C-terminal part of the glycoprotein B showed a higher detection rate and seems to be the immunodominant region with more epitopes for antibody-binding compared to the N-terminal part of the glycoprotein B.

6. Göttingen minipigs are often used for various biomedical investigations and are well tested concerning the presence of numerous bacteria, fungi, viruses, and parasites. The prevalence and expression of porcine endogenous retroviruses and the prevalence of HEV in Göttingen minipigs have already been investigated in our group. In publication VI the presence of PCMV and PLHV and an extended testing for HEV have been described. Using highly sensitive methods, PCMV, HEV, and PLHV were found in some Göttingen minipigs. The results show that highly sensitive methods are required to characterize pigs to be used for xenotransplantation in order to prevent virus transmission.

7. In publication VII the testing of PCV2 regarding safety in xenotransplantation was investigated. Göttingen Minipigs, Aachen Minipigs, genetically modified pigs generated for xenotransplantation and pigs from a slaughterhouse as well as pigs from a German farm were screened using a PCR method for PCV2. 50% of the Aachen minipigs and 14% of Göttingen minipigs were PCV2 positive, but the animals were apparently healthy. None of the slaughterhouse animals, the farm animals and the genetically modified animals were positive for PCV2, because they had been vaccinated. The data indicate that on the one hand PCV2 may be found in healthy pigs even under SPF conditions, and that a correct screening is indispensable for donor animals for xenotransplantation. On the other hand, vaccination is a powerful tool to prevent infection.

Expanding on the knowledge of these 7 publications, the development and establishment of highly selective and sensitive screening methods could be used to find safe and healthy donor animals for xenotransplantation studies in future. Moreover, these methods could be used to screen the recipients after transplantation studies and more data about putative transmission could be obtained. The best detection time points for transmission and material after a transplantation should also be examined. In addition, these methods are important for elimination programs of these viruses based on treatment with antiviral drugs, vaccination, Caesarean delivery, early weaning and embryo transfer. The data could help to improve and determine hygienic standards for international xenotransplantation guidelines. Using safe and healthy donor pigs, their own spf/dpf-facilities could be built up especially for xenotransplantation donor animals. Besides, generated PERV-inactivated primary cell lines using the CrispR/Cas9-gene editing method could be generated and analysed. It could be determined whether the eradication of copy numbers was successful or not. If the eradication was successful, the cell´s nuclei could be transferred via somatic cell nuclear transfer to produce PERV-free pig embryos and to generate PERV-free animals[96]. Furthermore, the detection of zoonotic pathogens in pigs for slaughter should gain attention, especially regarding the food chain. Hygienic standards should be improved

and pigs could also be tested on a random basis. Of course, other highly sensitive methods like ddPCR and deep sequencing should be used to prove the results obtained with the established real-time PCR methods.

Moreover, there are several other viruses which have been assumed to be zoonotic, because they can cause diseases in both, humans and animals. Nipah virus[211], Menangle virus[68], influenza virus A[73] or Rotavirus group C[212] are examples for other viruses to keep in mind, because it is known that they can cross the species barrier from pig to humans.

6 Literature

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Appendix

List of publications

1. Plotzki E, Wolf-van Buerck L, Knauf Y, Becker T, Maetz-Rensing K, Schuster M, Baehr A, Klymiuk N, Wolf E, Seissler J, Denner J.: *Virus safety of islet cell transplantation from transgenic pigs to marmosets*. Virus Res. 2015 Jun 2; 204:95-102.

2. Abicht JM, Mayr TA, Reichart B, Plotzki E, Güthoff S, Falkenau A, Kind A, Denner J.: *Hepatic Failure After Pig Heart Transplantation Into a Baboon: No Involvement of Porcine Hepatitis E Virus.* Ann Transplant. 2016 Jan 7; 21:12-6.

3. Plotzki E, Keller M, Ehlers B, Denner J.: *Immunological methods for the detection of porcine lymphotropic herpesviruses (PLHV).*J Virol Methods. 2016 Jul; 233:72-7.

4. Plotzki E, Heinrichs G, Kubícková B, Ulrich RG, Denner J.: *Microbiological characterization of a newly established pig breed, Aachen Minipigs.* Xenotransplantation. 2016 Mar; 23(2):159-67.

5. Plotzki E, Keller M, Ivanusic D, Denner J.: *A new Western blot assay for the detection of porcine cytomegalovirus (PCMV).* J Immunol Methods. 2016 Oct; 437:37-42.

6. Morozov VA, Plotzki E, Rotem A, Barkai U, Denner J.: *Extended microbiological characterization of Göttingen minipigs: porcine cytomegalovirus and other viruses.* Xenotransplantation. 2016 Nov; 23(6):490-496.

7. Heinze J, Plotzki E, Denner J.: *Virus safety of Xenotransplantation: Prevalence of porcine Circovirus (PCV2) in pigs.* Ann of Virol and Research. 2016 Dec

8. Fischer K, Kraner-Scheiber S, Petersen B, Rieblinger B, Buermann A, Flisikowska T, Flisikowski K, Christan S, Edlinger M, Baars W, Kurome M, Zakhartchenko V, Kessler B, Plotzki E, Szczerbal I, Switonski M, Denner J, Wolf E, Schwinzer R, Niemann H, Kind A, Schnieke A.: *Efficient production of multi-modified pigs for xenotransplantation by 'combineering', gene stacking and gene editing.* Sci Rep. 2016 Jun 29; 6: 29081.

List of posters

1. Elena Plotzki, Uwe Fiebig, Joachim Denner (2016), Virological safety in xenotransplantation-Development of new screening methods, SFB Meeting München

2. Elena Plotzki G. Heinrichs, B. Kubíckova, R.G. Ulrich, J.Denner (2016), Virological characterization of a newly established pig breed, Aachen minipigs **(Vortrag),** GFV Münster

3. Elena Plotzki, Martina Keller, Daniel Ivanusic, Bernhard Ehlers, Joachim Denner (2016), New immunological methods for the detection of porcine herpesviruses, GFV Münster

4. Elena Plotzki, Marwan Semaan, Daniel Ivanusic, Joachim Denner (2015), Transplantation tissue safety – screening and attempts for knock out of multiple porcine endogenous retrovirus (PERV) sequences in the pig genome, MPI Symposium Berlin

5. Elena Plotzki, Vladimir A. Morozov, Bernhard Ehlers, Joachim Denner (2015), Virological safety in xenotransplantation: Screening pigs for porcine herpesviruses, GFV Bochum

Eigenständigkeitserklärung

Die dieser Dissertation zugrunde liegenden Arbeiten wurden im Zeitraum vom

01. Juli 2014 bis 30. Juni 2017 am Robert-Koch Institut in Berlin angefertigt.

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet habe.

Berlin, den 22.09.2017 _____

Elena Janich geb. Plotzki