Animal Ethics and Zoonosis Risks

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Introduction

Organ transplantation is commonly utilized in people who suffer from end-stage organ failure [1]. In the United States (U.S.), on average, 17 people die every day from the lack of available organs for transplant [2]. Almost 106,000 people are currently on the waiting list for an organ transplant [3]. Kidneys, livers, hearts and lungs, in that order, are the most commonly transplanted organs [4]. However, the supply of human donor organs for transplantation is limited. While the number of living donor kidney and liver transplants continues to increase, the vast majority of organ transplant procedures involve organs from deceased donors. The U.S. saw a 6% increase in deceased donors, from 11,870 in 2019 to 12,588 in 2020 [3]. Hence efforts have been made to use animal organs in human patients, in a process called "xenotransplantation".

Xenotransplantation is defined as any procedure that involves the transplantation, implantation or infusion of either (a) live cells, tissues or organs from a nonhuman animal source or (b) human body fluids, cells, tissues or organs that have had *ex vivo* contact with live nonhuman animal cells, tissues or organs into a human recipient [2, 5, 6]. Ideally, the donor organ size is similar to humans and this limits the selection of suitable donor species. Animal species most compatible with the size requirement for humans include pigs, cattle and non-human primates [7].

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Organs obtained from non-human primates are closest and most similar to human organs. Macaques, baboons, squirrel monkeys, owl monkeys, and marmosets are species most commonly used in research facilities [8]. Limitations often include time-to-maturation, the length of gestation and the number of offspring (Table 8.1).

In the past, a major problem with xenotransplantation was hyperacute xenograft rejection i.e. the body recognizes the organ as non-self and mounts an immune response. Advances in technologies such as somatic cell nuclear transfer, viral transduction of DNA and use of CRISPR/Cas (clustered regularly interspaced short palindromic repeats, CRISPR; CRISPR-associated proteins, Cas) has allowed for humanization of non-human xenograft tissues [9]. In fact, the first human heart xenograft was from a genetically modified pig and was successfully completed in January 2022 at the University of Maryland Medical Center [10].

Since the global coronavirus (COVID-19) pandemic, declared on March 11, 2020 [11], the number of xenotransplantation procedures dropped by 90.6% in France and 51.1% in the U.S. [12]. COVID-19 is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This virus was first identified on January 9th 2020 in a patient in Wuhan, China [13]. The origin of SARS-CoV-2 has been ascribed to wild animals that harbored the virus and subsequently transmitted it to humans [14] though this has not been definitively determined. Interestingly, since wide spread testing has been implemented, many species of animals have tested positive for the SARS-CoV-2 infection including felines, canines, and some animals such as mink and have become prime case studies for zoonosis and reverse zoonosis with SARS-CoV-2 [15]. This pandemic demonstrated that the potential dangers of transmitting known or unknown pathogens through xenotransplantation are substantial and as such exercising utmost caution is prudent. However, we are at present equipped with very powerful tools to enhance our understanding and assessing the risks of zoonotic infections during xenotransplantation.

Table 8.1 Comparison of factors related to offspring in animal species considered suitable for xenotransplantation

	Non-human primates					
	Macaque *Macaca fascicularis*	Baboon *Papio hamadryas*	Squirrel monkey *Saimiri sciureus*	Marmoset *Callithrix jacchus*	Cattle *Bos taurus*	Pig *Sus domesticus*
Female sexual maturity (years)	1238 days (3.4 years)	1514 days (4.1 years)	1003 days (2.7 years)	477 days (1.3 years)	548 days (1.5 years)	152–182 days (0.5 years)
Gestation period	165 days	171 days	161 days	144 days	277 days	115 days
Inter-litter interval (litters per year)	431 days (0.8 or every 1.2 years)	568 days (0.6 or every 1.5 years)	365 days (1)	169 days (2)	365 days (1)	156 days (2.3)
Average number of offspring/ pregnancies	1	1	1	2	1	10–15

Animal Ethics

General Consideration

Within xenotransplantation animal ethics is an increasingly important topic [16–19]. Concerns over animal usage for the purpose of xenotransplantation include animal welfare issues, usage of genetic engineering, biosafety, and the rights of the animals themselves. Furthermore, as an overall ethical issue, there is a widespread belief, primarily for religious reasons, that certain areas in genetic engineering such as manipulating animals for human usage should not be studied [20].

Animal Welfare

Today pigs are an important source of animal protein for people globally. They are raised in a variety of environments including modern confinement facilities with carefully monitored environmental conditions as well as alternative production practices such as pasture rearing. Welfare regulations are in place but may differ across pork production areas and systems. These regulations often include measurements such as number of pigs per m^2, defined areas of continuous solid floor in contrast to slatted floor, minimal and maximal temperatures which are achieved by ventilation, dry bedding areas for the pigs to move to, defined lighting and noise levels, and the ability to express natural behavior among others. Becoming a donor for xenotransplantation puts pigs under different welfare regulations. In general, biosafety protocols are often in place to isolate the donor pigs from acquiring common infections. In addition, these pigs may be subjected to individual and unnatural housing conditions (including no bedding material and often restraining the natural behavior of pigs), surgical procedures, artificial insemination, *in vitro* fertilization, embryo transfer, cesarean derivation and colostrum deprivation. In addition, the donor pigs are subjected to regular and frequent sampling procedures by non-invasive or invasive methods, necessitating manual or drug induced restraint. Thus, breeding of pigs and rearing to obtain tissues for xenotransplantation will likely impact their welfare and natural behaviors. Animals being kept under "research" conditions that fail to meet the needs dictated by the animals' biological and psychological nature is a significant concern for society today and may create additional concerns in the future.

Genetic Engineering of Animals for Xenotransplantation

If non-human tissues are transferred into a human there is a high risk of an immunological rejection of the organ as the human immune system recognizes the foreign organ as "not-self" and rejects it. In what is known as "hyper-acute rejection", the body begins to reject the organ as soon as it is implanted [21]. Transplantation of organs requires lifelong immunosuppression of the recipient,

which is associated with significant morbidity [22, 23]. This disparity has fueled intense interest focused on alternative organ sourcing and regenerative medicine. As a solution for this problem, interspecies chimeras have been created which aid in the generation of humanized organs. Several advancements in this area benefited from new technologies, including genome editing tools, such as zinc finger nucleases, transcription activator-like effector nucleases (TALEN), and CRISPR/Cas9 technologies [24]. Today the genome of pigs can be more easily manipulated resulting in multiple gene knockouts, human transgene insertions, and more recently, specific animal organ knock outs and replacement with a humanized organ [24]. For example, greater than 6-month survival of a life-supporting kidney co-transplanted with a vascularized thymic graft into non-human primates has been achieved [25]. This could indicate that a hybrid thymus in combination with immunosuppression may prolong pig xenograft lifespans [25]. Similarly, triple gene knockout pigs have been developed for renal transplants to reduce the reactivity of pre-existing anti-pig antibodies in pre-transplant patients [26]. Humanized pigs certainly can also have major disadvantages. Possible problematic pre-existing anti-pig antibodies and methods to stop these from becoming a problem have been reviewed [27]. A cytidine monophosphate-n-acetylneuraminic acid hydroxylase and glycoprotein, alpha1, 3-galactosyltransferase double knockout pig model has been produced to reduce immune reactions during xenotransplantation in the human recipient [28]. However, the so humanized pigs were found to suffer from clinical signs and pathologic lesions such as swollen liver and spleen, increased deposition of hemosiderin and severe bleeding due to the genetic engineering [28]. Concerns with genetic engineering include suffering of the created chimeric animals [20].

Animal Rights

Due to the need to have a defined health status, animals raised for xenotransplantation often live in confined research facilities with little or no interaction with other pigs [29] compromising its right to express natural behavior. In Europe, the law dictates five freedoms for farmed animal: (1) Freedom from hunger and thirst, (2) freedom from discomfort, (3) freedom from pain, injury and disease, (4) freedom to express normal behavior, and (5) freedom from fear and distress. As already discussed in the section “Animal Welfare,” many if not all of these freedoms and rights are not available to these animals raised in laboratories. Animal rights questions may also arise if an animal is eventually selected and sacrificed to provide a xenograft for a human organ recipient. In contrast, if the animal is not selected ore viable for xenotransplantation, for example due to incompatibilities or birth deformities, such animals may be destroyed which poses ethical questions. Due to food safety regulations, genetically engineered pigs currently cannot enter into a regular food supply chain and likely would be culled and incinerated. Hence their existence may be considered a waste.

Alternative Approaches to Usage of Animals

Human–animal chimeras produced through various techniques, including stem cell biotechnology, regenerative medicine, and blastocyst complementation may offer alternatives to usage of live pigs [30]. Typically, pig receptors are changed to human receptors in transgenic pigs. However, it has been shown that two of three human complement regulatory proteins are also receptors for human viral pathogens: CD46 is the cell-surface receptor for measles virus, and CD55 can serve as a binding receptor for Echo and Coxsackie B picornaviruses [31]. Coxsackie B virus causes myocarditis and might endanger the pig heart in an immunosuppressed recipient of a xenograft. It could also pose a risk to the pig directly if infected by staff working in the research facility [31]. Generation of organs by 3D printing technology and decellularized scaffolds *in vitro* is currently available but not quite ready for usage [30, 32]. A simple approach to 3D-printing, thick, vascularized, and perfusable cardiac patches, created by using the patient's own cells, that completely match the immunological, cellular, biochemical, and anatomical properties of the patient has been published in 2019 [33]. This may become an alternative to xenotransplantation in the future.

Xenotransplantation and Possible Impact of Pig Viruses

As bacteria and parasites are commonly controlled by antimicrobials or antiparasitic drugs, for the purpose of this review only viruses will be discussed (Table 8.2). Initial research on xenotransplantation was conducted with organs from non-human primates, which are the closest phylogenetic and evolutionary relatives to humans. Concerns of transmission of pathogens from non-human primate organs to humans, such as the transmission of Herpes B virus discovered in 1932 [41], Ebola virus, first recognized in 1976 [42], the Marburg virus discovery in 1967 [43] and human immunodeficiency virus [44, 45] are felt to be too great to continue to consider the use of non-human primate organs. The use of non-human primates for xenotransplantation was banned due to the perceived high risk of zoonotic infections by the U.S. Food and Drug Administration in 1999 [46]. The risk of transmission of infection from other donor species, such as pigs – currently the most popular source for xenografts to humans, is also a concern albeit at a lower level. However, pigs can harbor a wide variety of different viruses (Table 8.2) and extensive diagnostic workup may be needed to confirm absence of potential harmful viruses to humans.

Pig heart valves are already routinely used in humans [47]. For cell transplantations, pig pancreatic islets may benefit human recipients with diabetes [48–50]. The most commonly used U.S. organ transplants include kidney, liver, heart, lungs, pancreas and intestines, whereas commonly transplanted tissues are bones, tendons, ligaments, skin, heart valves, blood vessels and corneas [51, 52]. Currently pig kidneys and possibly hearts, due to the fact that heart disease has remained the leading cause of death at the global level for the last 20 years, are the most common organs

Table 8.2 Overview of selected viruses in pigs

Virus family	Structure	Genus	Viruses in pigs	Abbreviation	Disease manifestation	Site of replication in the pig with emphasis on xenotransplantation importance			Zoonotic potential
						Viremia	*Heart*	*Kidney*	*Replication in humans or in human cell lines*
Adenoviridae	dsDNA, linear	*Mastadenovirus*	Porcine adenovirus 1–5	PAdV-1-5	Enteric, respiratory, systemic	+			No evidence
Arteriviridae	ssRNA (+) linear env	*Betaarterivirus*	Betaarterivirus suid 1 and 2 (formerly porcine reproductive and respiratory syndrome virus)	PRRSV	Reproductive, systemic, respiratory	+	+	+	No evidence
Asfarviridae	dsDNA, linear, env	*Asfivirus*	African swine fever virus	ASFV	Systemic, skin, enteric	+	+	+	No evidence
Anelloviridae	ssDNA (−) circular	*Iotatorquevirus* *Kappatorquevirus*	Torque teno sus virus 1a, 1b, k2a, k2b	TTSuV1a, 1b, k2a, k2b	Subclinical	+			No evidence
Astroviridae	ssRNA (+) linear	*Mamastrovirus*	Porcine astrovirus 1–5	PAstV1-5	Enteric, CNS (PAstV3 only)	Unclear/rare			Unclear, suspected
Bunyavriridae	ssRNA (−or+) linear, env	*Orthobunyavirus*	Akabane virus		Subclinical	+			No evidence
		Not classified	Oya virus		Subclinical	+			No evidence
			Lumbo virus Tahyna virus		Subclinical	+			Zoonosis
		Herbevirus	Herbert virus	HEBV	Subclinical				Unknown
		Goukovirus	Gouleako goukovirus	GOLV	Subclinical				Unknown
Caliciviridae	ssRNA (+) linear	*Norovirus*	Porcine norovoris	PoNoV	Subclinical				No evidence
		Sapovirus	Porcine sapovirus	PoSaVs	Enteric	+			No evidence
		Unassigned	St-Valérien calicivirus	SVCV	Subclinical	+			No evidence
		Vesivirus	Vesicular exanthema of swine virus	VESV	Systemic, vesicular lesions				No evidence

Circoviridae	ssDNA (−) circular	*Circovirus*	Porcine circovirus 1, 2, 3, 4	PCV1, 2, 3, 4	Enteric, systemic, respiratory, reproductive	+	+	+	No evidence in humans, low level of PCV2 infectivity in cancerous and normal human cell lines[a]
Coronaviridae	ssRNA (+) linear, env	*Alphacoronavirus*	Swine acute diarrhea syndrome coronavirus also known as swine enteric alphacoronavirus	SADS or SeACoV	Enteric	+			No evidence in humans but several primary human lung cell types and primary human intestinal cells are susceptible [34]
			Transmissible gastroenteritis virus	TGEV	Enteric	+			No evidence
			Porcine respiratory coronavirus	PRCV	Respiratory	Unclear/rare (2 dpi)			No evidence
			Porcine epidemic diarrhea virus	PEDV	Enteric				No evidence
		Betacoronavirus	Porcine hemagglutinating encephalomyelitis virus	PHEV	Respiratory, CNS, systemic	+	+	+	No evidence
		Deltacoronavirus	Porcine deltacoronavirus	PDCoV	Enteric	+			No evidence in humans but human hepatoma (Huh7) cells and cells from other animal species are susceptible [35]
		Torovirus	Porcine torovirus	PToV	Subclinical				No evidence
Filoviridae	ssRNA (−) linear env	*Ebolavirus*	Reston ebolavirus	RESTV	Subclinical	+			Zoonosis
			Zaire ebolavirus	EBOV	Subclinical	+			Zoonosis

(continued)

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						Viremia	*Heart*	*Kidney*	*Replication in humans or in human cell lines*
Flaviviridae	ssRNA (+) linear env	*Flavivirus*	Japanese encephalitis virus	JEV	Subclinical, reproductive	+			Zoonosis
			Murray Valley encephalitis virus	MVEV	Subclinical	+			Zoonosis
			West Nile virus	WNV	Subclinical	+			Zoonosis
		Pestivirus	Atypical porcine pestivirus	APPV	Reproductive, CNS	+			No evidence
			Border disease virus	BCVV	Reproductive, respiratory				No evidence
			Bovine diarrhea virus	BVDV	Reproductive				No evidence
			Bungowannah virus		Reproductive	+			No evidence
			Classical swine fever virus	CSFV	Reproductive, skin, respiratory, systemic	+	+	+	No evidence
Hepeviridae	ssRNA (+)	*Orthohepevirus*	*Hepatitis E virus*	HEV	Subclinical	+			Zoonosis

Herpesviridae	dsDNA (+) linear env	*Macavirus*	*Ovine gammaherpesvirus 2* or Malignant catarrhal fever (Ovine herpesvirus 2)	OvHV-2	Ocular discharge, CNS, systemic				No evidence
			Suid gammaherpesvirus 3, 4 and 5 (Porcine lymphotrophic herpesvirus 1, 2 and 3)	PLHV 1–3	Subclinical	+			No evidence
		Roseolovirus	Suid betaherpesvirus 2 Porcine roseolovirus better known as porcine cytomegalovirus)	PRV or PCMV	Subclinical, respiratory	+			Zoonotic event during xenotransplantation
		Varicellovirus	Suid alphaherpesvirus 1 (Pseudorabies virus)	PRV	Respiratory, CNS	+		+	No evidence, humans are resistant
Orthomyxoviridae	ssRNA (−) linear env	*Influenza virus A*	Influenza A virus—swine	IAV-S	Respiratory				Zoonosis
		Influenza virus B	Influenza B virus	IBV	Respiratory				Zoonosis
		Influenza virus C	Influenza C virus	ICV	Respiratory				Zoonosis
		Influenza virus D	Influenza D virus	IDV	Respiratory				No evidence
Paramyxoviridae	ssRNA (−) linear env	*Rubulavirus*	Menangle virus		Reproductive				Zoonosis
			Blue eye paramyxovirus or La Piedad-Michoacan virus	BEPV or LPMV	CNS, corneal opacity, reproductive				No evidence
		Henipavirus	Nipha virus	NiV	Respiratory, CNS	+			Zoonosis
			Hendra virus		Respiratory				Zoonosis
		Respirovirus	Porcine parainfluenza virus 1	PPIV1	Respiratory				No evidence

(continued)

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						Viremia	*Heart*	*Kidney*	*Replication in humans or in human cell lines*
Parvoviridae	ssDNA (−) linear	*Parvoviruses*	Porcine parvovirus 1–7	PPV1–7	Reproductive, skin	+	+		No evidence in humans or in human cell lines [36]
Picornaviridae	ssRNA (+) linear	*Cardiovirus*	Encephalomyocarditis virus	EMCV	Sudden death		+		Zoonosis
		Aphthovirus	Foot and mouth disease virus	FMDV	Vesicular lesions, salivation, systemic	+	+	+	No evidence
		Enterovirus	Porcine enteroviruses	PEV	Skin				No evidence
		Kobuvirus	Porcine kubovirus	PKoV	Reproductive, enteric	+			No evidence
		Unassigned	Porcine picornavirus Japan	PPVJ	Subclinical				No evidence
		Sapelovirus	Porcine sapelovirus	PSV	Reproductive, enteric, CNS	+			No evidence
		Teschovirus	Porcine teschovirus	PTV	Subclinical, CNS	Unclear/rare			No evidence
		Senecavirus	Seneca Valley virus	SVV	Skin, fever, lameness	+			No evidence
		Pasivirus	Swine pasivirus A1-A3		Unknown, CNS?				Unknown
		Enterovirus	Swine vesicular disease virus	SVDV	Vesicular lesions, systemic				No evidence but likely
Poxviridae	dsDNA linear env	*Suipoxvirus*	Swinepox virus	SWPV	Skin				No evidence

Reoviridae	dsRNA segmented	*Rotavirus*	Rotavirus	RV A, B, C, E and H	Enteric	+			No evidence but RV A recombination with human RV has been observed [37] and human RAV can infect pigs [38]
		Orhoreovirus	Reovirus	ReoV	Enteric, respiratory, CNS				No evidence but human-bat transmission likely
Retroviridae	ssRNA (+) linear env	*Gammaretrovirus*	Porcine endogenous retrovirus A, B, C	PERVA-C	Subclinical	+			No evidence
Rhabdoviridae	ssRNA (−) linear env	*Lyssavirus*	Rabies virus	RV	CNS, salivation				Zoonosis
		Vesiculovirus	Vesicular stomatitis virus	VSV	Salivation, vesicular lesions	Unclear/rare			Susceptible
Togaviridae	ssRNA (+) linear env	*Alphavirus*	Eastern equine encephalitis virus	EEEV	CNS				Zoonosis
			Getha virus	GETV	CNS, enteric, reproductive, subclinical				No evidence
			Sagiyama virus	SAGV	Subclinical				No evidence

[a] Cancerous human cell lines (MCF-7, A549, HeLa, HepG2, U937, THP-1) and normal human cell lines (293T, WI-38, HUVEC, WISH, HSAS4, HEH2) [39, 40]

of interest to be transplanted into humans. Because of this, any virus that may replicate in kidneys or the heart is currently of most concern. In addition, many viral infections cause viremia i.e. presence of viruses in the blood and hence such viruses can be found at times in the kidneys, heart or any other organ.

In a landmark surgery, a porcine heart from a genetically modified pig was transplanted to a 57-year-old man with severe heart failure on January 7, 2022 at the University of Maryland School of Medicine [10]. The recipient's condition started suddenly deteriorating 40 days after the transplantation surgery and eventually the patient died on March 8, 2022. On April 20, 2022, during a webinar of the American Society of Transplantation, the surgeon who conducted the xenotransplantation announced the potential role of a porcine cytomegalovirus (PCMV) infection in the death of the recipient. An extremely low level of PCMV virus was detected in the recipient on the 20th day after the xenotransplantation and the virus levels became precipitous by the 40th day, potentially contributing to the recipient's deterioration. The PCMV is a herpes (DNA) virus in the genus *Roseolovirus* which can go into latency [53]. Though the highly genetically modified donor pig, supplied by a private company, was raised under stringent conditions to avoid infections and was screened for multiple pathogens, the latent infection with PCMV was not detected. Later analysis detected the PCMV in the donor pig's spleen tissue. This single event highlights the importance of zoonotic infections, including latent ones, in xenotransplantation.

A virus transmitted through xenotransplantation could evolve to be transmitted to other humans, potentially causing a wider outbreak and thus this event could pose an ethical quandary. Interestingly, concerns about xenotransplantation and a negative impact of PCMV were first raised in 2015 due to the observation that transplantation of PCMV contaminated pig organs into non-human primates was associated with a significant reduction of the survival time of the transplants [54]. PCMV is related to human cytomegalovirus and human herpesviruses 6 and 7 which can cause serious disease among immunocompromised human individuals, including transplant recipients [55]. The author suggested that the pathogenicity of PMCV may be due to disruption of the coagulation system and suppression and exhaustion of the immune system. Hence, PCMV should be eliminated from donor pigs despite the lack of knowledge on replication of the virus in human cells [55]. In a follow-up study, the distribution of PMCV in baboon organ recipients, who received PCMV contaminated hearts, was investigated [56]. Interestingly, PCMV antigen (as demonstrated by immunohistochemistry) was present in cells in all of the organs of two baboon recipients despite indications that herpes viruses are species-specific. In addition, the same research group also detected PCMV in several organs of the donor pigs that had not been detected in blood when tested at an earlier time point, indicating that testing blood is not an efficient way to detect PCMV in young pigs [56]. In another study, it was found that PCMV transmission in orthotopic pig heart xenotransplantation was associated with a reduced survival time of the transplant and increased levels of interleukin (IL) 6 and tumor necrosis factor (TNF)α were found in the baboon recipient [57]. Furthermore, high levels of tissue plasminogen activator (tPA)-plasminogen activator inhibitor type 1 (PAI-1) complexes were found, suggesting a complete loss of the pro-fibrinolytic properties of the

endothelial cells. These data show that PCMV has an important impact on transplant survival and emphasizes the importance for elimination of PCMV from donor pigs [57]. Based on these findings and the need to prevent PCMV transmission during xenotransplantation, new diagnostic nested and real-time PCR methods have been developed [58]. It has been suggested to use early testing of oral and rectal swabs by uniplex real-time PCR [59]. In addition to viral nucleic acid, a Western blot assay for detection of PCMV antibodies in donor pig candidates has also been described [60]. Early weaning at 24 hours after birth and removal of the dams from a newly established pig donor facility completely eliminated PCMV [61]. Alternatively, immunosuppression of the donor pigs to reactivate PCMV may also need to be considered in future.

Under experimental settings, porcine organs are transplanted into non-human primates for research purposes, and the personnel working on these projects are directly exposed to the experimental animals. This scenario leads to multiple risks of cross species infections involving all three species, which could potentially evolve and spill over to other animals and/or the general human population. Besides well-known pathogenic viruses there are numerous viruses that do not cause clinical signs. This group is divided into viruses that are recognized and may be monitored and viruses that are not recognized and hence are not monitored routinely. An example of a virus that falls into the first group is swine flu; the presence of asymptomatic viral swine infections potentially compatible with humans and not part of routine pig veterinary screening is a great concern for xenotransplantation. Pathogens that may fall into the latter group include porcine endogenous retrovirus (PERV), porcine astrovirus (PAstV), herpesviruses including PCMV and others. It has been shown previously that infectious complications are a major cause of morbidity and mortality after heart transplantation from human-to-human [62]. Among 113 patients included in the study, 92 (81%) patients developed at least one infection within 180 days after heart transplantation among which viral infections were diagnosed in 44 (34%) patients and involved mostly cytomegalovirus infection (n = 39, 34%) [62].

General Concepts on Pig Health Status and the Impact of Pig Derivation and Housing

Pig Health Status

As a general rule, a viral infection in a pig can result in a subclinical infection (no clinical signs, the pig appears healthy) or in clinical disease. Clinical disease can be further subdivided into different levels of severity (mild, moderate, severe) with different durations (acute, chronic, persistent). Clinical signs can vary considerably and can be suggestive of respiratory viruses (e.g. sneezing, nasal discharge, coughing), enteric viruses (e.g. diarrhea, lack of appetite, vomiting), systemic viruses (neurological signs, fever, lethargy) and others. The virus propagation at one point peaks and then declines. Once antibodies against the virus are produced, viremia/shedding becomes intermittent and eventually the virus is no longer detectable for most viruses.

Table 8.3 Definitions of pig types that can be procured and their expected virus status

Pig type	Housing		Caesarean section	Colostrum access after birth	Possible virus exposure	
	Dam	*Piglet*			*Vertical*	*Horizontal*
Conventional	Farm	Farm	No	Yes	Transplacental Birth canal passage	Litter mates Environment
CD[a]	Farm	Farm 1h→Exp.[b]	No	No	Transplacental Birth canal passage	Litter mates Environment
CDCD[c]	Farm/ Exp.	Exp.[b]	Yes	No	Transplacental	Littermates Environment
Gnotobiotic[d]	Farm/ Exp.	Exp.[b]	Yes	No	Transplacental	No

[a]Colostrum-deprived [63]
[b] Experimental unit or research facility
[c] Caesarean-derived-colostrum deprived pig
[d] Raised germ free

Pig Derivation and Housing Impacts Circulating Viruses

The overall number of pathogens and specifically the viruses or virus load in a pig ultimately depends on how the pig is derived, reared and housed. There are major differences in pig derivation (Table 8.3) and also in housing. In general, pigs used for research and transplants are often caesarean derived (birth by C-section) and may or may not be colostrum deprived. They are typically housed in biosecurity level 2 (BSL-2) or even BSL-3 units with direct contact to care staff or may be raised in gnotobiotic chambers. Gnotobiotic pigs are derived by C-section directly into a sterile chamber and reared with no direct contact to humans and fed sterilized food [64]. Often such high health pigs are housed in high efficiency particulate air (HEPA)-filtered, negative pressure facilities under biosecurity level 2 (BSL-2) or BSL-3.

Conventional High Health Pig Farm

Considerably different from gnotobiotic or caesarean-derived-colostrum deprived (CDCD) pigs, pigs can be sourced from a "high health herd". These pigs are typically raised in modern commercial confinement facilities and are documented to be free of certain pathogens. These herds are commonly monitored by surveillance testing and they may or may not utilize viral and bacterial vaccines. If a pig source is negative for certain pathogenic viruses it is often classified as having a high health status or specific pathogen free (SPF). However, high health or SPF status is not equivalent to being free of all pathogenic viruses or bacteria. Economically important pig viruses, based on geographic region and location, that are commonly tested for in pigs from high health farms include porcine reproductive and respiratory syndrome virus (PRRSV), influenza A virus (IAV), porcine circovirus type 2 (PCV2) and others.

Virus Transmission in Pigs to Assess the Potential of Introducing Viruses into Secure Research Facilities

Direct Pig-to-Pig Transmission or Vertical Transmission from the Dam to the Intrauterine Offspring

The direct transmission, also known as horizontal transmission, results from direct contact of infected and non-infected pigs on a farm and depends on virus shedding routes and the shedding duration. For example, PCV2, a ubiquitous pig virus, can be shed via various routes including nasal secretions, saliva, feces, urine, colostrum or semen [65] and the length of viremia has been determined to be up to 140 days [66]. On the other hand, vertical transmission is when the virus crosses the placental barrier and starts replicating in the endometrial and/or placental tissues. For some viruses including PRRSV [67, 68], PCV2 [69, 70], PPV [71, 72], vertical transmission is very important. Intrauterine virus infection of fetuses with any of these viruses often results in fetal death and abortion or mummification; however, pigs may also be born alive, often suffering from myocarditis [73], being more susceptible to other pathogens and may serve as virus source for other pigs.

Indirect Transmission

Different vectors such as insects and birds [74], contaminated fomites including shoes, clothing, feed [75] and others can also contribute to virus spread between pigs and farms. It has also been shown that airborne transmission of viruses [76, 77] is possible between different pens, barns, and even farms [78]. Some viruses can survive for extended time periods under favorable conditions such as organic material, high humidity, low UV light and low temperatures [79–81].

Viruses in Pigs

Virus Populations in a Pig

Table 8.2 includes a list of relevant pig viruses. However, it needs to be noted that at any given time, a pig harbors a number of organisms, including viruses, bacteria and parasites, which are important for normal day-to-day functions but can also result in disease. Virus infections in pigs can be divided into notifiable diseases, reportable diseases, economically important diseases and viruses of currently unknown importance. Next generation sequencing efforts have resulted in discovery of a large number of viruses in pigs [82] for which the importance in health and disease is largely unknown. Often no clinical signs have been associated with these viruses and further testing to understand their replication or prevalence are not commonly done.

Known Zoonotic Viruses

Per definition a zoonosis is an infectious disease that has jumped from a non-human animal to humans and includes viruses (further listed below), bacteria or parasites.

Lumbo Virus and Tahyna Virus

In pigs, members of the *Bunyaviridae* family including Lumbo virus and Tahyna virus are considered zoonotic but are not associated with clinical signs in pigs. Both viruses are widespread in some human populations with occasional clinical consequences. The role of pigs in the bunyavirus ecology is largely unknown [83, 84].

Reston Ebolavirus and Zaire Ebolavirus

Other well-known zoonotic viruses that can also infect pigs include Reston ebolavirus and Zaire ebolavirus both from the *Filoviridae* family. For the Reston ebolavirus, pig-to-human transmission has been confirmed [85]. Typically, pigs do not develop clinical signs [85].

Japanese Encephalitis Virus (JEV), Murray Valley Encephalitis Virus and West Nile Virus (WNV)

Within the *Flaviviridae* family several members are zoonotic including JEV [86, 87], which is distributed across most of Asia, the western Pacific and northern Australia. Clinical signs in pigs are often not evident despite increased numbers of stillborn and mummified fetuses. Murray Valley encephalitis virus [88, 89] is enzootic in the Kimberley region of Western Australia and in parts of the Northern Territory. The virus is epizootic in regions further south in Western Australia and the southern half of the Northern Territory. Finally, the WNV within the *Flaviviridae* is commonly found in Africa, Europe, the Middle East, North America and West Asia and also causes subclinical infection in pigs [90].

Hepatitis E Virus (HEV)

In pigs HEV was first detected in 1997 in the U.S. [91]. Today it is recognized that the virus is present in all major pork producing areas and infection of a pig is essentially always subclinical with most pig herds infected [92]. When pork products (particularly pork liver) are consumed raw, zoonotic transmission to humans can occur [93].

Influenza Viruses

It has been demonstrated that influenza A virus (IAV) can be transmitted from humans to pigs. A few pig-to-human transmissions of IAV are reported each year; however, evidence of onwards infection in humans is limited [94]. In contrast, influenza B virus (IBV) infections occur mainly in humans and are rare in pigs [95, 96]. A similar scenario is also true for influenza C virus (ICV) which is rare in pigs [97].

Influenza D virus (IDV) has been identified in pigs in 2011 [98] and this virus does not seem to occur frequently.

Menangle Virus, Nipha Virus and Porcine Parainfluenza Virus 1 (PPIV1)

In the family *Paramyxoviridae* there are several zoonotic viruses that can infect pigs including Menangle virus which has been reported in outbreaks in pigs in Australia, in Malaysia and Singapore. Pigs are considered amplifying hosts for Nipha virus which causes severe disease in humans [99]. Hendra virus is distributed in Africa and Australia. Experimental infection of pigs with Hendra virus resulted in mild respiratory symptoms [100]. Finally, PPIV1 has been demonstrated to replicate in experimentally infected pigs and induced mild respiratory signs [101]. There is no confirmed evidence of a zoonotic transmission of PPIV1 to humans; however, there is a high similarity with the human virus version.

Rabies Virus

Another well-known zoonotic disease in pigs is rabies virus which results in clinical disease in infected pigs [102]. Rabies is relatively rare in pigs and is characterized of a sudden onset of salivation, rapid chewing, muscle spasms, and aggression. Typically pigs die within 3 days.

Eastern Equine Encephalitis Virus

This virus from the family of *Togaviridae* causes clinical signs in pigs ranging from incoordination, depression, vomiting and mortality which is most evident in pigs less than 2 month of age. Occasionally there are outbreaks [103]. Virus distribution is in North, Central and South America.

Pig Viruses of Importance to Xenotransplantation

In addition to zoonotic viruses there are viruses circulating in pigs that are thought of as being pig specific but could pose a high risk to human transplant recipients.

Porcine Circoviruses (PCV)

Pigs are commonly infected with PCVs including PCV1, PCV2, PCV3 and PCV4 [104]. While there are disease manifestations associated with PCV2 and less frequently PCV3, pigs are commonly subclinically infected [105]. PCV2 is immunosuppressive [106] in pigs but it is currently unknown if a PCV infection could impair pig transplant functionality. In addition, vaccination against PCV2 is able to prevent PCV-associated disease in pigs; however, in most cases not transmission of the virus. Therefore, PCV2 has to be eliminated to obtain xenografts from uninfected healthy animals [106]. Even though circoviruses from pigs are

commonly found in human stool samples [107, 108], to this date, disease in people has not been observed even when PCV1 and PCV2 were transmitted by contaminated rotavirus vaccines to children as determined from vaccine trials containing data from more than 100,000 children [107, 109]. While a study showed that PCV1 DNA could be detected in feces of infants up to 36 days after vaccination, the authors concluded that the levels of PCV1 DNA detection were more supportive of virus passage in the gastrointestinal tract than replication [110]. Furthermore, there was no evidence of seroconversion to PCV1 in infants 1–2 months post administration of an oral rotavirus vaccine containing live PCV1 [111]. Although there is evidence that PCV2 does not infect at least immunocompetent-humans, donor animals should be screened using sensitive methods and ensure virus elimination by selection, caesarean delivery, vaccination, or embryo transfer [106].

Porcine Lymphotrophic Herpesvirus (PLHV) and Porcine Cytomegalovirus (PCMV)

Within the *Herpesviridae* family there are two genera with potential to infect humans via xenotransplantation. There is no evidence currently that PLHV can infect humans, although a recent review indicated that there is a great potential [112]. In contrast, for PCMV pig-to-primate [113] and pig-to-human transmissions [114] have been confirmed.

Encephalomyocarditis Virus (EMCV)

Within the *Picornaviridae* family, EMCV which as the name implies causes inflammation in the heart, is thought to have a low risk for being zoonotic; however, it may be of great importance for xenografts [115]. The virus is known to persist [116] and has been proven to transmit to mice during xenotransplantation of infected tissues from pigs [117]. Natural infection has been confirmed in *Macaca sylvanus* and *Hystrix cristata* from an Italian rescue center [118].

Swine Vesicular Disease Virus (SVDV)

The enterovirus SVDV is similar to human coxsackievirus B5 (CS-B5), in fact SVDV is a variant of CS-B5 [119] and both cause similar lesions. Host switching events have been reported [120].

Porcine Endogenous Retroviruses (PERV)

PERVs are yet another group of viruses which are causing concerns for xenotransplantation. Unlike regular viruses, which can be removed by rigorous strategies, PERVs are part of the cells of pigs. PERVs have been previously reviewed [121] and gamma and beta retroviruses have been found integrated into the genome of pigs [115]. Sequencing of the entire pig genome revealed 212 PERV insertions in the genome [115]. The gamma retroviruses include PERV-A and PERV-B, which are integrated into the genome of all pigs, and PERV-C, found in many (but not all) pigs. Ways to inactivate PERVs such as via CISPR-Cas9 [122, 123] are being investigated. In previous pig-to-small animal or pig-to-non-human primate

transplantation trials testing the impact of pharmaceutical immunosuppression, PERV replication was not upregulated [124, 125].

Cross Species Transmission Using the SARS-CoV-2 Example

General Concepts and Definitions

Most viruses normally have a narrow host range. Cross-species viral transmission describes a process by which a virus successfully infects (productive infection) a new host species and subsequently adapts to it. This process is also known as host jumping or spillover. Xenotransplantation recipients are often immunosuppressed and thus their immune system is not acting at full strength to fight pathogens. This opens a window of opportunity for a non-human pathogen to adapt to its new human host and spread to other humans.

The SARS-CoV-2 Pandemic: An Example for Virus Cross-Species Transmission

The ongoing COVID-19 pandemic associated with SARS-CoV-2, was initially observed with severe respiratory disease in a cluster of patients in Wuhan, Hubei Province, China during December 2019 [13]. The causative agent, a novel coronavirus (2019-nCoV), later re-named SARS-CoV-2, was identified and consequently reported to the World Health Organization [13] On 11-March-2020 the World Health Organization (WHO) upgraded SARS-CoV-2 infection to a global pandemic [11]. Overall the spread of SARS-CoV-2 in humans has been remarkable. A pool of 7.753 billion SARS-CoV-2 naïve people were present when the virus started infecting humans. Based on data from the U.S. and eight European countries it has been determined that the early epidemic grew exponentially at rates between 0.18 and 0.29 per day (epidemic doubling times between 2.4 days and 3.9 days) [126]. The virus spread with high speed through most countries and continents resulting in a high number of infected people that shed virus for extended periods of time. As people naturally have close relationships with pets, it was not surprising that cross-species transmission was reported on 28 March 2020 in a Belgian cat who belonged to a person confirmed infected with SARS-CoV-2 [127]. This was then followed by detection of the virus in other animals. SARS-CoV-2 was diagnosed. on two mink farms (designated NB1 and NB2) in the Netherlands on 23 and 25 April 2020, respectively [128]. A requirement for successful SARS-CoV-2 replication in humans but ultimately also of non-human species is having the correct angiotensin-converting enzyme 2 (ACE2) which serves as functional receptor for the spike protein of SARS-CoV-2. The ACE2 receptor is widely distributed in animals and has a protective role in the cardiovascular system and in alveolar epithelial cells [129]. Adaptive mutations in the viral genome can alter the virus's pathogenic potential. Even a single amino acid exchange can drastically affect the ability of a virus to

evade the immune system and complicate the vaccine development progress against the virus [130]. The receptor-binding domain (RBD) in the virus is therefore essential. Once the importance of ACE2 for human invasion of SARS-CoV-2 was realized, numerous studies have focused on identifying animal species that may have ACE2 receptors similar to humans and hence may be at higher risk to become a reservoir for the virus [131]. In silico structural homology modelling, protein–protein docking, and molecular dynamics simulation study of SARS-CoV-2 spike protein's ability to bind ACE2 from relevant species indicated the highest binding to human ACE2 with the next highest binding affinity to pangolin ACE2 whereas the affinity of monkey ACE2 was much lower [132]. Other ACE2 species in the upper half of the predicted affinity range (monkey, hamster, dog, ferret, cat) have been shown to be permissive to SARS-CoV-2 infection, supporting a correlation between binding affinity and infection susceptibility [132]. Similarly, other studies confirmed these results and predicted that the ACE2 receptor from animals such as dogs, tigers, camels, cats, dwarf hamsters, and sheep have a slightly increased affinity to SARS-CoV-2-RBD [133].

Cross-Species Transmission of SARS-CoV-2 from Humans to Animal Species

Shortly after SARS-CoV-2 entered and adapted to the human population, case reports started to be published indicating human-animal transmissions of virus. Initially this involved mainly indoor pets including cats [127] and dogs [134] living in close contact with COVID-19 affected owners. Later these findings were experimentally confirmed and SARS-CoV-2 infection was also found in other species [135].

Farmed Mink

Mink have a high susceptibility to SARS-CoV-2 infection. The first farm with SARS-CoV-2 infection in mink occurred in the Netherlands and was reported on 26-April-2020 [136]. During the outbreak investigation a few important things were found: (1) The mink were likely infected with SARS-CoV-2 through close contact with human care staff (Fig. 8.1), (2) the mink developed severe clinical respiratory disease and transmission within the farm happened fast, (3) an investigation in to the SARS-CoV-2 virus circulating in the mink revealed that while there was close relationship to the human SARS-CoV-2 strain, mutations had already occurred most likely as consequence of adjustment to the host, and (4) the adapted mink-SARS-CoV-2 strain was found in care takers indicating a true species jump from humans to mink and back into humans [137]. Shortly after finding the virus in Dutch mink, the virus was also found in farmed mink in Spain, Denmark, USA, Italy, Sweden, Greece, France, Poland and Lithuania. While further research indicated that the SARS-CoV-2 mutants in mink did not increase fitness in the human airway [138], the fast spread of the virus in mink and its adaption to its new host species resulted in large culling effort on mink farms [139].

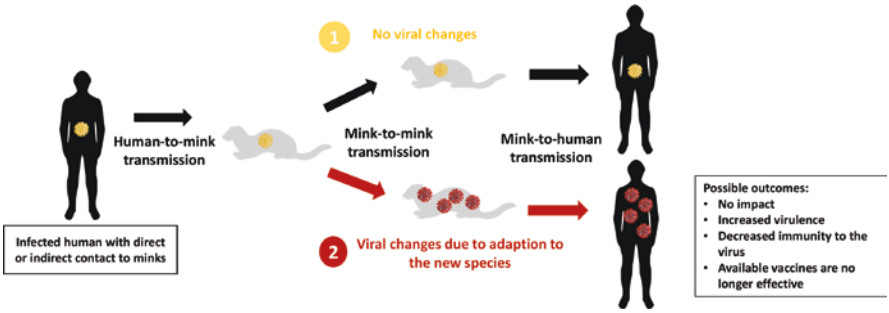

Fig. 8.1 Possible scenarios viruses undergo during cross species transmission with the example SARS-CoV-2 and human-to-mink transmission and subsequent mink-to-human transmission

Pigs

Several experimental studies on SARS-CoV-2 susceptibility were done using pigs [140–142]. The overall result indicated that pigs have a very low susceptibility to the virus. Initially, shortly after SARS-CoV-2 was discovered, a surveillance study conducted in China was not able to find any SARS-CoV-2 antibodies in 187 randomly selected pigs from commercial farms [143]. Several experimental challenge studies in pigs followed. Investigators used different challenge strains and different virus doses to infect the pigs; however, most studies could not confirm active virus replication, seroconversion or transmission [135, 144]. A Spanish study demonstrated seroconversion in experimentally infected pigs but the investigators were unable to find replicating virus in any of the pigs [141]. A U.S. study found no evidence of clinical signs, viral replication or SARS-CoV-2-specific antibody responses in nine 5-week-old pigs when infected through the oral, intranasal and intratracheal routes. However, the same study also found that porcine cell lines including a porcine kidney cell line and swine testicular (ST) cell line could be readily infected [142]. In contrast, a Canadian study using sixteen 8-week-old pigs inoculated with SARS-CoV-2 via an oronasal route found low susceptibility to infection in these pigs based on detection of viral RNA in nasal wash (2/16 pigs at 3 days post challenge) and pooled oral fluids (1/2 at 3 days post challenge), as well as the successful isolation of virus from a pig [145]. Furthermore, 2/16 pigs developed neutralizing antibody titers against SARS-CoV-2 between 11-days and 15-days post challenge [145]. Hence there appears to be a very low risk of pigs getting infected and developing an established active infection.

Summary and Conclusions

During xenotransplantation humans receiving donor organs or tissues are frequently immune suppressed for various time periods and therefore vulnerable to infectious diseases. Creating human-pig chimeras could be a major advantage as human organs are extremely limited. However, there could potentially be great risks as virus populations of two different host species would be mixing in a person with a suppressed immune system (Fig. 8.2).

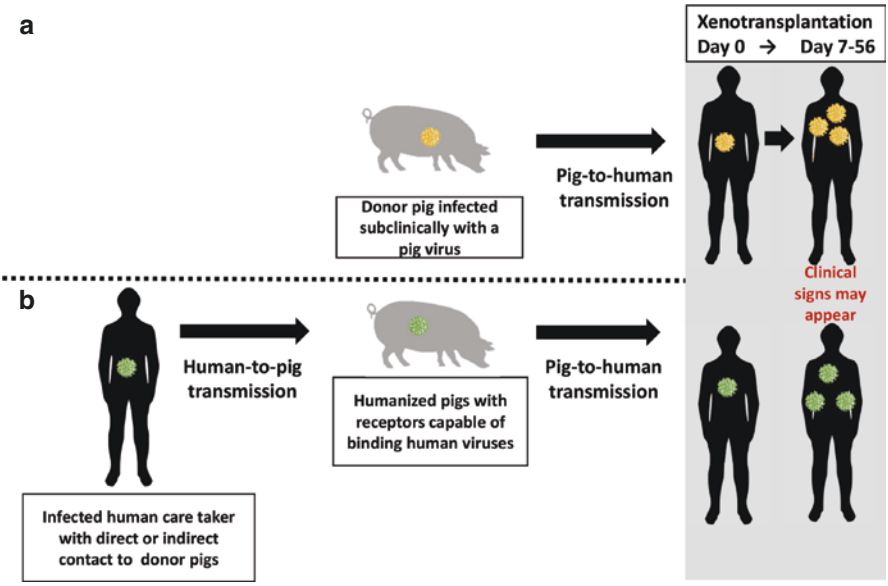

Fig. 8.2 Possible scenarios for cross species transmission of viruses during xenotransplantation using pigs (**a**) Presence of a pig pathogen in a pig organ donor and (**b**) presence of a human pathogen in a care staff, transmitted into the donor pig population due to presence of human cell receptors in the humanized pigs. In both cases, the virus will be transferred into the immunosuppressed human organ recipient with likely consequences to the donated organ and organ recipient but also possible onwards human-to-human spread

Scientists working in xenotransplantation need to work closely with scientists working with animals, particularly with pigs. A constant exchange of the latest knowledge on the ecology of donor pig viruses, a mutual understanding of the use of the best detection methods for these viruses and the limitations of these tests needs to occur to be as sure as possible that the donor pig is free of infectious agents. Essentially all of the latest molecular diagnostic techniques used in human medicine are also available today in veterinary diagnostic medicine and in many cases those techniques are used more routinely in modern pork production than in human health. For example, veterinary diagnostic laboratories such as the Iowa State University Veterinary Diagnostic Laboratory [146] routinely offer a menu of individual PCR assays and PCR panels for swine diseases, conduct next generation and whole genome sequencing and have a large number of serological assays available.

The SARS-CoV-2 observations in species other than humans has provided concern and insight into the ability of emerging viruses to jump species and spillover into the human population. The first pig-to-human heart transplant patient likely died of myocarditis due to a common pig pathogen (PMCV). Especially for the xenotransplantation application, methods to activate dormant/latent/quiescent viruses to replicate to detectable levels needs to be investigated. For instance, corticosteroids or immunomodulators or specific agents could be

utilized and if the method has been defined and is working, they may provide a way to activate silent or non-detectable viruses so that they replicate and are detected by screening tests.

Overall, the tremendous need for donor organs should drive advancement in science to effectively confirm that pigs are free of viruses that could potentially harm the human donor recipient. This is a great opportunity for collaboration between clinicians and researchers in animal and human health.

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