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# Recent Progress in Xenotransplantation, with Emphasis on Virological Safety

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Xenotransplantation is a new technology that may help to overcome the shortage of human tissues and organs available for the treatment of tissue and organ failure. Remarkable progress has recently been made in this field. First, understanding of the mechanisms of immunological rejection, mainly of the hyperacute rejection, allowed generating numerous genetically modified pigs to overcome rejection. Second, based on these genetically modified animals and new immunosuppression regimens, long-term survival of non-human primate recipients of heart, kidney, and islet cell cells has been reported. And third, potential zoonotic microorganisms have been identified in pigs and sensitive methods to detect them have been generated. In 2 clinical trials treating diabetic patients with porcine islet cells, no porcine microorganisms were transmitted to human recipients. Furthermore, strategies to eliminate potentially zoonotic microorganisms from donor pigs in order to prevent transmission to the recipients have been developed, including designated pathogen-free (DPF) breeding. In addition, strategies to prevent transmission of porcine endogenous retroviruses (PERVs) have been developed, including a knockout of all proviruses in the pig genome by gene editing. PERVs are integrated in the genome of all pigs and therefore they cannot be eliminated by DPF breeding. Since they are able to infect human cells, they represent a special risk in xenotransplantation. Despite the achievements, some problems remain: numerous genetically multi-modified pigs have been generated without fully evaluating their advantage, and microbiological screening of pigs to be used for transplantations and elimination of pathogenic microorganisms from the donor pigs are still not satisfactory.

**MeSH Keywords:** **Microbiological Safety • Transplant Rejection • Transplant Survival • Xenotransplantation**

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## Background

The shortage of human organs for the treatment of tissue and organ failure is well known. There are several ways to cope with this: prevention of diseases leading to organ failure, increase of the number of human donors, and implantable electromechanical devices, which have been explored in the field of cardiac transplantation. However, these strategies will not be a solution for most of the patients on the waiting list. An alternative would be to create vascularized organs from stem cells, which is currently far from reality. Another solution could be xenotransplantation, which is transplantation of cells, tissues, or organs from another species. Pigs are for many reasons (e.g., size, physiology, large number of progeny, and genetic modification) the favored donor species. Xenotransplantation using pig cells, tissues and organs has to overcome 3 hurdles before being applied in the clinic (Figure 1): immunological rejection, physiological incompatibility, and risk of transmission of potentially pathogenic microorganisms, which may induce a zoonosis. Microbiological safety is very important and porcine endogenous retroviruses (PERVs) are of special interest because they are integrated in the genome of all pigs and cannot be eliminated (like many other potentially zoonotic pig microorganisms) by specified or designated pathogen-free (SPF or DPF) breeding. Here we summarize the recent successes in the field.

## Immunological Rejection

In comparison to allotransplantation, the immunological rejection of pig tissues and organs is much more complex. The first step of immunological rejection in xenotransplantation is hyperacute rejection (HAR), which is well studied. HAR is based on pre-existing antibodies directed mainly against galactose-α 1,3-galactose [Galα13Galβ1- [3]4GlcNAc-R, α-gal] epitopes. The α-gal epitope is abundantly synthesized on glycolipids and glycoproteins of non-primates and New World monkeys by the glycosylation enzyme α1,3galactosyltransferase (α1,3GT) [1]. In humans, apes, and Old World monkeys, this epitope is absent because the α1,3GT gene was inactivated in ancestral Old World primates. Instead, humans, apes, and Old World monkeys produce anti-Gal antibodies, which specifically interact with α-gal epitopes and which constitute ~1% of circulating immunoglobulins [1]. Since α-gal epitopes are present on the surface of bacterial cells and cells from other animals, the antibodies directed against the α-gal epitope represent a powerful tool to protect against bacterial infection, as well as from the transmission of foreign cells. When anti-Gal antibodies interact with pig cells, they activate complement and induce the rejection of the pig transplant [2]. Based on this knowledge, transgenic animals were created, overcoming the rejection process. There are 2 ways to prevent rejection: by a knockout of the cellular enzyme α1,3GT (GTKO pigs), which is adding α-gal

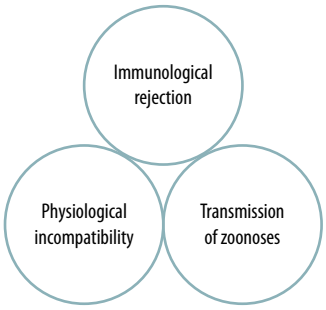


**Figure 1.** Xenotransplantation: Main hurdles.

epitopes to pig cell surface molecules [3], and by expression of human complement-regulatory proteins such as hCD46, hCD55, and hCD59 on pig cells (for review, see [4]). Animals expressing different combinations of the complement-regulatory proteins have been created (for overview, see Table 2 in [5]). In addition, immune T cell responses were reduced by simultaneous expression of CTLA4-IgG and mutant MHC class II transactivator [6–9].

α-gal epitopes and 2 N-glycoylneuraminic acid-terminated (Neu5Gc) gangliosides are targets for pre-existing antibodies in human sera [10]. The expression of Neu5Gc is also specific for pigs and some other animals, but not for humans, because the corresponding enzyme, cytidine monophosphate-N-acetylneuramic acid hydrolase (CMAH), was inactivated during primate evolution [11]. Double-knockout pigs deficient in α-gal and Neu5Gc significantly reduce the humoral barrier to xenotransplantation [11]. Using zinc finger nuclease (ZFN) technology, GGTA1/CMAH knockout pigs were produced [11], which were characterized by a reduced binding of human antibodies to transgenic peripheral blood mononuclear cells (PBMCs) *in vitro* [12]. In lung perfusion experiments, hCD46 expression in GTKO pigs down-regulated complement activation, diminished platelet and coagulation cascade activation, neutrophil sequestration, and histamine release [13]. Altogether, transplants from these genetically modified pigs are characterized by significantly reduced rejection.

When HAR is averted, xenotransplants become subject to acute vascular rejection (named because of its similarity to acute vascular rejection in allotransplantation), also called acute humoral xenotransplant rejection (AHXR) or delayed xenotransplant rejection. Expression of human proteins involved in endothelial activation, such as heme oxygenase 1 (HO-1) [14] or tumor necrosis factor-induced human protein A20 [15], as well as of human antithrombotic or anticoagulant genes such as tissue factor pathway inhibitor (TFPI), endothelial protein C receptor (EPCR), or thrombomodulin (TM), may also enhance xenotransplant survival [16]. In the case of islet preparations, cellular reactions usually play the dominant role in islet xenotransplant



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rejection. Blocking T cell activation by antigen-presenting cells (APC) via interfering with the co-stimulatory systems CD40-CD40L and/or CD80/86-CD28 could be utilized to reduce this reaction [17–21]. In a pig-to-humanized mouse transplantation, transgenic porcine islet cell clusters expressing the T cell costimulation blocking molecule LEA29Y normalized blood glucose levels of diabetic mice and were, in contrast to wild-type porcine islets, protected against rejection by human immune cells [22]. T cell activation is regulated by co-stimulatory as well as by co-inhibitory receptor-ligand interactions. Enhancing inhibitory signals by transgenic expression of respective ligands on porcine cells is an attractive new concept to diminish human anti-pig cellular immune responses [23]. The observation that immune responses to pig cells overexpressing the human inhibitory ligand PD-L1 (CD274) are particularly weak *in vitro* and *in vivo* supports the relevance of this approach [24,25]. It was proposed that combining blockade of co-stimulatory signalling pathways (e.g., by CTLA-4.Ig/LEA29Y) with an enhancement of inhibitory signals by targeting the PD-1/PD-L1 pathway should be highly effective in controlling cell-mediated rejection of xenotransplants [26]. Regulatory T cells (Tregs) represent another approach to inhibit T cell-mediated rejection [27,28]. In addition, natural killer (NK) cells, macrophages, and neutrophils are critical components of the cellular response in xenotransplant rejection [29–31]. For example, expression of HLA-E, an inhibitor of NK cell activation, has been found to protect porcine cells from destruction by primate NK cells [32]. T cell responses against the xenotransplant are closely associated with immediate blood-mediated inflammatory reaction (IBMIR) [33, 34] and the resulting activation of complement factors and coagulation [34].

Numerous genetically-altered pigs have been generated, but their real advantage for the survival of pig cells, tissues, and organs has not been evaluated.

## Increased Survival Times

Due to the newly generated genetically modified pigs and new and effective immunosuppressive treatments preventing antibody and immune T cell responses, a prolonged survival of the transplants was observed. A comprehensive overview of the experimental transplantations in the years until 2013 is given by Cooper et al. [35]. To demonstrate the development in the field, the longest survival times listed by Cooper et al. [35] and survival times published later [6,36–43] are summarized in Tables 1 and 2. The ability to modify pigs genetically to protect the donor organs from the primate's immune response has resulted in survival of heterotopic pig hearts in baboons for longer than 2.5 years [39–41,44,45] (Table 1). Increase in immunosuppression, but not in anticoagulation, improved heterotopic GTKO pig heart survival in baboons [46].

In contrast to the heterotopic heart xenotransplantations, the survival time of orthotopic xenotransplantations is much shorter (at present, up to 57 days; Table 1). In this context it seems useful to remember the initial survival times in the first clinical heart allotransplantations; the first recipient lived for 18 days and the second for 19 months (for a historical overview, see [47]).

Life-supporting pig kidneys survived for almost 3 months in NHP [48, 49] (Table 1). Expression of human complement pathway-regulatory proteins, hCD46 or hCD55, in combination with GTKO reduced the incidence of early transplant failure (loss of function within 3 days of transplantation) to 7%, but did not prevent systemic coagulation activation [50]. Encouraging results were obtained when a GTKO pig transgenic for CD46, CD55, thrombomodulin (TBM), endothelial proteins C receptor (CD39), and blood type 0 donor was used. Although the expression of CD39 and TBM was low, a kidney from this animal transplanted under an effective immunosuppressive therapy and using anti-inflammatory agents was functional for 136 days [8, 42] (Table 1). A survival time of over 125 days was observed when kidneys from CTKO/CD55 pigs were transplanted and the recipients were treated with anti-CD154 monoclonal antibodies [41]. Experience with pig liver xenotransplantation, however is sparse, with maximum graft survival in the NHP of only 9 days [51–53].

## Physiological Compatibility

At present, the physiological compatibility is difficult to evaluate since the survival time of the transplanted organs is usually too short to analyze the long-term functionality of the transplant and its interaction with the transplant recipient in detail. When the physiological aspects of pig to non-human primate renal xenotransplantation were studied using organs from pigs transgenic for human decay accelerating factor (hDAF, hCD55), the porcine kidneys largely maintained plasma electrolyte homeostasis, but an increase in proteinuria and severe anemia were detected in the recipient [54]. The anemia, often observed in renal pig to non-human primate xenotransplantation, could be related to the inability of porcine erythropoietin to adequately stimulate primate hematopoietic precursors, and treatment of the recipient with exogenous human erythropoietin will be required. Alternatively, the generation of transgenic pigs producing sufficient amounts of human erythropoietin may solve the problem.

More difficult is the situation with pig liver transplantation [55]. In pig to non-human primate studies, the transplantation of livers from pigs transgenic for hCD55 or from GTKO animals, which in addition expressed hCD46, was associated with a survival time of 7 to 9 days [55–57]. Although hepatic functions,


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**Table 1.** Xenotransplantation of organs, longest survival times, up to April 2016.

| Recipient (number of animals) | Transgenes | Immunosuppressive regimen | Longest survival time (median) days | Reference |
|---|---|---|---|---|
| **Heterotopic heart transplantation** | | | | |
| Baboon (14) | CD55 | Cyp, CsA, CS, MMF | 99 (26) | Bhatti et al., 1999 [116] |
| Baboon (10) | CD46 | ATG, splenectomy, anti-CD20mAb, tacrolimus, rapamycin, CS, TPC | 113 (76) | McGregor et al. 2004 [117] |
| Baboon (10) | CD55 | ATG, anti-CD20mAb, TI, CVF, anti-CD154mAb, MMF, CS | 139 (27) | Houser et al., 2004 [118] |
| Baboon (7) | CD46 | ATG, splenectomy, anti-CD20mAb, tacrolimus, rapamycin, CS, TPC | 137 (96) | McGregor et al., 2005 [119] |
| Baboon (13) | CD46 | splenectomy, anti-CD20mAb, tacrolimus, rapamycin, TPC; heparin + ATG, CyP for rejection episodes | 109 (18) | Byrne et al., 2005 [120] |
| Baboon (8+2) | GTKO or low expression | ATG, TI, anti-CD2mAb, anti-CD154mAb, CVF, MMF, methylprednisolone* | 179 (78) | Kuwaki et al., 2005 [44] Tseng et al., 2005 [45] |
| Baboon (63) | CD46 | Splenectomy, anti-CD20mAb, tacrolimus, rapamycin, TPC; aspirin/clopidogref or lovenox or warfarin | 139 (96) | Byrne et al., 2006 [46] |
| Baboon (7) | GTKO, CD46, TBM or CD55 | Anti-CD20mAb, heparin** | 130 | Iwase et al., 2015 [43] |
| Baboon (5) | GTKO, CD46, TBM | ATG, anti-CD20mAb, CVF, anti-CD40mAb, MMF, CS | 945 (298) | Mohiuddin et al., 2013 [392] Mohiuddin et al., 2016 [41] |
| **Orthotopic heart transplantation** | | | | |
| Baboon (10) | CD55 | CyP, CsA, CS | 9 | Schmoeckel et al., 1998 [21] |
| Baboon (2) | WT | Immunosadsorption, TBI, CsA, methotrexate | 18, 19 | Xu et al., 1998 [122] |
| Baboon (1) | CD55 | CyP, CsA, MMF, CS | 39 | Vial et al., 2000 [123] |
| Baboon (4) | CD55 | ATG, tacrolimus, rapamycin, CS, GAS914, CyP | 25 | Brandl et al., 2005 [124] |
| Baboon (4) | CD55 | CyP, CsA, CS, MMF | 20 (14.6) | Brenner et al., 2005 [125] |
| Baboon (13) | CD55/CD46 | ATG, CyP, tacrolimus, rapamycin, CS, GAS914 | 25 | Brandl et al., 2007 [126] |
| Baboon (14) | CD46 or CD55 or GTKO/CD55 | ATG,or CyP, tacrolimus, rapamycin, anti-CD20mAb | 57 (6) | Byrne et al., 2011 [127] |
| **Kidney** | | | | |
| Cynomolgus (9) | CD55 | CyP, CsA, CS, splenectomy | 78 (39) | Cozzi et al., 2000 [128] |
| Cynomolgus (7) | CD55 | CyP, CsA, MMF, CS | 90 (48) | Baldan et al., 2004 [128] |



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**Table 1 continued.** Xenotransplantation of organs, longest survival times, up to April 2016.

| Recipient (number of animals) | Transgenes | Immunosuppressive regimen | Longest survival time (median) days | Reference |
|---|---|---|---|---|
| Baboon (5) | GTKO | Thymokidney, anti-CD2mAb, WBI, thymectomy, anti-CD154mAb | 83 (26) | Yamada et al., 2005 [49] |
| Baboon (7) | GTKO | Thymectomy, splenectomy, TBI, ATG, anti-CD2mAb, anti-CD154mAb, tacrolimus, MMF | 83 (49) | Griesemer et al., 2009 [130] |
| Baboon (4) | GTKO | ATG, anti-CD2mAb, anti-CD154mAb, MMF, CS, thymectomy, spelectomy, thymokidney, | 83 (52) | Shimizu et al., 2012 [131] |
| Baboon (5) | CD55 | thymokidney, thymectomy, splenectomy, immunoadsorption, anti-CD2mAb, anti-CD154mAb, ATG, tacrolimus, CyP, CVF, MMF, CS | 229 (27) | Barth et al., 2003 [132] |
| Rhesus (5) | GTKO/CD55 | Anti-CD4, anti-CD8, anti-CD154mAb, MMF, steroids | >133 (6–>133) | Higginbotham et al., 2015 [41] |
| Baboon (1) | GTKO/CD46/ CD55/TBM/ EPCR/ blood type 0# | ATG, anti-CD20mAb, CVF, anti-CD40mAb, rapamycin, MP## | 163 | Iwase et al., 2015 [42] |

* Supportive therapy: prostacyclin, dopamine, ganciclovir, levofloxacin, cimetidine, heparin, antithrombin, aspirin; ** two regimens were used, first ATG+anti-CD154mAb+CTLA4-Ig, second ATG+anti CD40mAb+CTLA4-Ig; # hTBM and hCD39 were not expressed in the kidney; ## in addition anti-inflammatory (tocilizumab, IL-6 receptor blockade, etanervept, TFN-α antagonist) and adjunctive (aspirin, low molecular weight heparin) treatment. ATG – anti-thymocyte globulin; CD46 – membrane cofactor protein; CD55 – decay-accelerating factor; CS – corticosteroids; CsA – cyclosporine; CTLA4-Ig – cytotoxic T-lymphocyte-associated protein 4 coupled to immunoglobulin; CVF – cobra venom factor; CyP – cyclophosphamide; EPCR – endothelial protein C receptor, CD39; GAS914 – a soluble glycoconjugate comprising Gal on a poly-L-lysine backbone; GTKO – α1,3-galactosyltransferase gene-knockout; MMF – mycophenolate mofetil (or analog, e.g. mycophenolate sodium]; MP – methylprednisolone; TBI – total body irradiation; TBM – thrombomodulin; TI – thymic irradiation; TPC – an αGal-polyethylene glycol polymer conjugate.

including coagulation, have proved to be satisfactory, the immediate development of thrombocytopenia was extremely limiting. The thrombocytopenia resulted in hemorrhages in various organs and tissues, as well as in the transplanted liver [56].

Barriers to successful lung transplantation appear to be even greater than for other organs [58]. They may be related to anatomical factors such as the fragile lung parenchyma-associated blood supply, as well as the presence of large numbers of inflammatory cells. The longest survival of a pig lung after transplantation in a non-human primate has been 5 days [58]. In contrast to the longer survival times of heart and kidney transplantations, lung transplantations are comparable with liver transplantation, which reached the longest survival of 9 days [57].

## Microbiological Safety

As already described in the introduction, PERVs are of special interest when analyzing the microbiological safety of xenotransplantation. Like endogenous retroviruses from other species, including humans, they are part of the genome and therefore cannot be eliminated, as in many other potentially zoonotic microorganisms in pigs, by SPF or DPF breeding [5]. PERV-A and PERV-B are in the genome of all pigs; PERV-C is common, but not present in all pigs. PERV-A and PERV-B are able to infect human cells, but PERV-C infects only pig cells. However, recombination between PERV-A and PERV-C resulted in recombinant PERV-A/C viruses, which are characterized by a very high replication rate. PERV-A/C were not found to be integrated in the germ line.

To date, there has been no reported transmission of PERVs in more than 200 individuals who had contact with pig tissues, either through islet cell transplantation or *ex vivo* perfusion of porcine livers and spleens. PERVs were also not transmitted in preclinical pig to non-human primate transplantations, or in infection experiments with small animals or non-human primates with or without pharmaceutical immunosuppression (for a review, see [5]). However, most of the individuals were not exposed for a long time to the xenotransplants and, with some exceptions (associated with parallel kidney allotransplantations), no immunosuppression had been applied. Non-human primates are not a suitable model in which to study the risk of PERV transmission because they (in contrast to humans)


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**Table 2.** Xenotransplantations of islet cells with the longest survival times, up to July 2015.

| Number of transplantation | Recipient (number of animals) | Transgenes (GE)/ Encapsulation (E) | Immunosuppressive regimen | Longest survival time (days) | Reference |
|---|---|---|---|---|---|
| 1 | Cynomolgus (2) | E | None | >256 | Elliott et al., 2005 [133] |
| 2 | Cynomolgus (4) | wt | Anti-CD25mAb, FTY720, rapamycin, anti-CD154mAb, tacrolimus, rapamycin, CS, TPC | 47 to 187 | Hering et al., 2006 [18] |
| 3 | Rhesus (9) | wt | Anti-CD25mAb, anti-CD154mAb, CTLA4Ig, rapamycin | 4 to >260 (140) | Cardona et al., 2006 [17] |
| 4 | Cynomolgus (9) | wt, GTKO, CD46 | ATG; anti-CD154mAb, MMF | 5 to 396 | Van der Windt et al., 2009 [134] |
| 5 | Cynomolgus (2) | wt | ATG; anti-CD25mAb, anti-CD20mAb, FTY720, rapamycin, CTLA4-Ig | 280, 380 | Hecht et al., 2009 [135] |
| 6 | Rhesus (9) | wt | Anti-CD25mAb, anti-CD40mAb, rapamycin, CTLA4-Ig | 47 to 203 (80) | Thompson et al., 2011 [136] |
| 7 | Rhesus (10) | GTKO or wt | Anti-CD25mAb, anti-LAF1mAb, MMF, CTLA4-Ig | 50 to 249 (137) | Thompson et al., 2011 [137] |
| 8 | Cynomolgus (6) | GTKO, CD46,CD39 | Anti-CD25mAb | 14 to 224 | Veriter et al., 2013 [138] |
| 9 | Cynomolgus (5) | 3GE – 4GE* | Complex treatment** | 0 to 365 (106) | Bottino et al., 2015 [139] |
| 10 | Rhesus (5) | wt | ATG, CVF, anti-CD154mAb, sirolimus | Up to 603 | Shin et al., 2015 [140] |

* 3GE, GTKO,CD46, CD39; 4GE, GTKO,CD46, TFPI, CTL4-Ig; 5GE, GTKO,CD46, TFPI, CTL4-Ig, CD39; ** Prostacyclin, methylprednisolon, dextran sulfate, ATG, MMF, anti-CD154mAb. Abbreviations see Table 1.

carry a mutated receptor for PERV, only allowing infection with reduced efficacy (reviewed in [59]). Despite the presence of a functional receptor for PERV on human cells, no transmission of PERV was observed in the first clinical trials transplanting pig islet cells to treat diabetes in New Zealand [60] and Argentina [61]. Therefore, the question of whether PERVs may be transmitted during xenotransplantation is still open and an elimination of infectious proviruses is advised.

In contrast to PERVs, transmission of herpesviruses has been observed in experimental xenotransplantations, e.g., porcine cytomegalovirus (PCMV) from pig to cynomolgus monkeys [62] and baboons [63]. Transmission of a baboon cytomegalovirus (BCMV) after transplantation of baboon tissues to human recipients was also described [64]. Most of the potentially zoonotic microorganisms can be eliminated by Caesarean section and SPF or DPF breeding of the animals [65]. However, there are viruses that may be transmitted via the placenta and therefore may be present even in SPF/DPF facilities [66,67]. At present, it is still unclear which porcine microorganisms have a zoonotic potential when transmitted to human recipients.

The following viruses are considered potentially pathogenic: PERVs, PCMV, hepatitis E virus (HEV), porcine lymphotropic herpesviruses (PLHV-1, -2, and -3), porcine circoviruses (PCV1 and PCV2), and others (Table 3). PERVs were included on this list because retroviruses in general induce tumors and/or immunodeficiencies [68]. Examples are human immunodeficiency virus 1 (HIV-1), inducing AIDS, and the human T cell lymphotropic virus 1 (HTLV-1), inducing lymphoma and neurological diseases in humans. HEV is on this list because it has been shown to be transmitted from pigs to humans, either by close contact or by undercooked pork, causing hepatitis in numerous cases [66,69,70]. PCMV is on the list because it is related to human cytomegalovirus (HCMV). HCMV infections are in many cases fatal for immunosuppressed allotransplant recipients [71]. Although PCMV is more closely related to HHV-6 and HHV-7 than to HCMV, designated now as HHV-5 [72], it has been shown that transmission of PCMV in pig to non-human primate kidney transplantation drastically reduced the survival time of the recipients [62,63], suggesting that PCMV may have a similar effect in humans. PLHV-1, -2, and -3 were found in large numbers on farm pigs (in 78%, 41%, and 59% of the



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**Table 3.** Potentially zoonotic viruses in pigs.

| Viruses | Zoonotic potential | Diseases in pigs |
|---|---|---|
| Porcine endogenous retrovirus (PERV) | Unknown | Unknown (tumour, immunodeficiency?) |
| Porcine cytomegalovirus (PCMV) | Yes | Rhinitis*, immunosuppression? |
| Porcine reproductive and respiratory syndrome virus (PRRSV) | No | PRRS; respiratory diseases in young pigs and reproductive diseases in sows |
| Porcine circovirus type 2 (PCV2) | Unknown | PCVD; PMWS |
| Hepatitis E virus (HEV) | Yes | Subclinical |
| Menangle virus | Yes | Reproductive disease |
| Porcine torovirus (PToV) | Unknown | Diarrhoea |
| Porcine sapovirus (porcine SaV) | Potential | Diarrhoea |
| Porcine lymphotropic herpesviruses (PLHV) | Unknown | Unknown but incriminated in PTLD |
| Nipah virus (NiV) | Yes | Respiratory and neurological syndrome |
| Torque teno sus virus (TTSuV) | Unknown | Unknown and PMWS |
| Bungowannah virus | Unknown | Porcine myocarditis syndrome |
| Porcine kobuvirus | Unknown | Unknown |
| Porcine bocavirus (PBoV) and other related novel porcine parvoviruses | Unknown | Unknown |

* Rhinitis in newborn piglets can be severe enough to cause haemorrhage from the nose. PRRS – porcine reproductive and respiratory syndrome; PCVD – porcine circovirus 2 diseases, previously; PMWS – post-weaning multisystemic wasting syndrome, PTLD – post-transplant lymphoproliferative disease.

lung tissue samples, and in 59%, 26% and 62% of the spleen samples, respectively [73]). Approximately 21% (9 of 44) of the miniature swine PBMC were also positive for PLHV DNA [74]. There is evidence that PLHV-1 is associated with post-transplant lymphoproliferative disease (PTLD) in miniature pigs following allogeneic hematopoietic stem-cell transplantation [75,76]. The clinical symptoms of experimental porcine PTLD, such as fever, lethargy, anorexia, high white blood cell count, and palpable lymph nodes, are similar to those of human PTLD, which was linked to the human herpesvirus Epstein-Barr virus, now designated HHV-4 [77]. Evidence of productive PLHV-1 infection was not detected in recipient baboons receiving different organs from transgenic pigs for up to 6 months of transplant function [78]. Appropriate breeding procedures can eliminate PLHV and piglets free of PLHV were produced via Caesarean section and barrier-reared breeding procedure [74]. In contrast to the porcine cytomegalovirus [PCMV], which can be excluded from source animals by early weaning of piglets, this was not possible in the case of PLHV [79]. With the exception of the PERVs, all potentially zoonotic viruses (Table 3) can be eliminated by SPF/DPF breeding. This includes Caesarean delivery and breeding in barrier facilities, and, if necessary, treatment of the pig herd with antiviral drugs [66,80]. Most important are the quality and sensitivity of the detection method; use of methods with low sensitivity could result in false-negative results.

Antiviral drugs have still not been used in pigs to treat infections. Ribavirin may be used for the treatment of HEV infections and ganciclovir, cidofovir, or other antiviral drugs for the treatment of PCMV infections, although PCMV – in contrast to the human CMV – is highly resistant to ganciclovir [81]. Combining treatment, selection, and (possibly) vaccination of the donor pigs, xenotransplantation can become a considerably safer technology compared with allotransplantation, in which transmissions of HIV-1, rabies virus, CMV, and other pathogens have been described [71].

To prevent transmission of PERVs, which are present in the genome of all pigs and cannot be eliminated by SPF/DPF breeding [5], several strategies have been developed. First, pigs can be selected that have a low copy number and a low expression at the RNA or protein level of PERV-A and PERV-B proviruses. Methods have been developed to discriminate between high and low expression of PERV in blood cells [82,83]. Second, PERV-C-free animals can be selected in order to avoid recombination between the ecotropic PERV-C (which infects only pig


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cells) and the human-tropic PERV-A. Such recombinant PERV-A/C are characterized by an increased replication competence compared with the parenteral virus [84–87]. Sensitive and specific methods have been developed to screen for PERV-C-positive animals [88,89]. Third, RNA interference technology has been successfully used in transgenic pigs to reduce the expression of PERV *in vitro* [90–92] and *in vivo* [93–95]. Fourth, vaccination is an excellent tool to prevent virus transmission. Immunizing with the transmembrane and surface envelope (TM and SU, respectively) proteins of PERV in different species binding and neutralizing antibodies were obtained, suggesting that this may also be possible in humans [96–98]. Fifth, since PERV is present in up to approximately 100 copies in the genome, it is a challenge to knock out all proviruses in the genome using gene editing. When gene editing was performed using the zinc finger nuclease (ZFN), the expression of ZFN was very high, inducing a toxic effect when the nucleases were cutting in multiple sites and destabilizing the genome [99]. When the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated (CRISPR/Cas9) technology [100–102] was used, a breakthrough was achieved. Sixty-two PERV proviruses were knocked-out in immortalized PK-15 pig cells [103]. If it will be possible to knock out all infectious PERV proviruses in primary cells and to obtain piglets not releasing PERV, these retroviruses are no longer a risk for xenotransplantation [104]. To summarize, efficient strategies have been developed to identify and to prevent transmission of porcine microorganisms with zoonotic potential, including PERVs.

## Ethical Aspects and Regulation

Although not a topic of this review, it should be mentioned that theological-ethical [105–107] and regulatory aspects of xenotransplantation are being broadly discussed. The International Xenotransplantation Association [IXA] recently published the first update of a consensus paper dealing with different aspects of efficient and safe islet cell xenotransplantation [108–115]. One contribution to this consensus paper provides a detailed description of national regulatory frameworks [109].

## Conclusions

Here, we demonstrated numerous steps forward to bring xenotransplantation towards clinical application. First of all, multitransgenic pigs were created in order to prevent rejection of the pig cells and organs. Second, new immunosuppression regimens were introduced, also in order to prevent rejection. Third, based on these achievements, longer survival times of transplanted pig hearts, kidneys, liver, and islet cells have been observed in preclinical trials. Fourth, new and sensitive methods have been developed to screen the donor pigs for potential zoonotic microorganisms, making xenotransplantation eventually safer compared with allotransplantation, where in rare cases HIV-1, rabies virus, HCMV, and other pathogens have been transmitted [71]. Fifth, the discussion on ethical aspects is ongoing, an updated consensus document on how to perform safe and efficient xenotransplantation was prepared by the scientific community, and in several countries a national regulatory framework was prepared. All these achievements will allow clinical application of xenotransplantation in the near future.

Two major fields in xenotransplantation research are in need of improvement. First, although pigs with multiple genetic modifications have been produced with great effort and expense, in most cases their advantage compared to other genetically modified pigs was not fully evaluated. Second, microbiological characterization of the donor pigs has, in the past, not been sufficiently thorough, and as a result, pig to non-human primate transplantations have been performed that resulted in the transmission of pathogenic microorganisms and the premature death of the recipients. For example, the transmission of PCMV to cynomolgus monkeys and baboons, significantly reducing survival time, has been reported [62,63]. Additional financial support and scientific investigations have to be devoted to studies of viral safety, the results of which should automatically contribute to increasing the survival times of xenotransplants and their recipients.

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