

The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes – Chapter 5: Recipient monitoring and response plan for preventing disease transmission

**Joachim Denner¹, Ralf R.Tönjes², Yasu Takeuchi³, Jay Fishman⁴,
Linda Scobie⁵**

¹Robert Koch Institute, Berlin, Germany, ²Paul Ehrlich Institute, Langen, Germany, ³Division of Infection and Immunity, University College, London, United Kingdom, ⁴Infectious Disease Division, Massachusetts General Hospital, Boston, USA, ⁵Glasgow Caledonian University, Glasgow, United Kingdom,

Abstract: Xenotransplantation of porcine cells, tissues and organs may be associated with the transmission of porcine microorganisms to the human recipient. A previous, 2009, version of this consensus statement focused on strategies to prevent transmission of porcine endogenous retroviruses (PERVs). This version addresses potential transmission of all porcine microorganisms including monitoring of the recipient and provides suggested approaches to the monitoring and prevention of disease transmission. Prior analyses assumed that most microorganisms other than the endogenous retroviruses could be eliminated from donor animals under appropriate conditions which have been called “designated pathogen-free” (DPF) source animal production. PERVs, integrated as proviruses in the genome of all pigs cannot be eliminated in that manner and represent a unique risk. Certain microorganisms are by nature difficult to eliminate even under DPF conditions; any such clinically relevant microorganisms should be included in pig screening programs. With the use of porcine islets in clinical trials, special consideration has to be given to the presence of microorganisms in the isolated islet tissue to be used and also to the potential use of encapsulation. It is proposed that microorganisms absent in the donor animals by sensitive microbiological examination do not need to be monitored in the transplant recipient; this will reduce costs and screening requirements. Valid detection assays for donor and manufacturing-derived microorganisms must be established. Special consideration is needed to preempt potential unknown pathogens which may pose a risk to the recipient.

This statement summarizes the main achievements in the field since 2009 and focus on issues and solutions with microorganisms other than PERV.

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Introduction

Xenotransplantation using pig materials may be associated with transmission of porcine microorganisms to the recipient. In general, most microorganisms could be eliminated by designated pathogen free (DPF) production of the donor animals which includes Cesarean section, closed containment, special precautions concerning feed and waste, excellent training of the staff and measures to prevent transmission of microorganisms from the staff to the herd. However, porcine endogenous retroviruses (PERVs) cannot be eliminated in this way since they are integrated in the genome of all pigs, and may produce virus particles which are able to infect some human cells *in vitro* [1]. It is important to note that only certain transformed human tumour cell lines can be infected by PERV derived directly from pig cells. However, after adaptation on human cells associated with genetic modifications, PERV also infects human primary cells *in vitro* [1, 2]. In the previous, 2009, version of this consensus statement [3], strategies to prevent PERV transmission were elaborated. A detailed analysis of risk posed by PERVs and the corresponding measures to prevent transmission was undertaken subsequently. In addition, other porcine microorganisms which could infect human recipients were studied and the risks posed by them were analyzed. Although they were thought to be eliminated easily by designated pathogen free production, difficulties were observed in generating pigs free of designated pathogens such as hepatitis virus E and herpesviruses [4-7]. In addition, better detection methods were developed to identify pigs free of these microorganisms [8, 9]. In general, if microorganisms are eliminated from the donor pig, there should be no need to continue to routinely monitor recipients for these specific microbes. With this approach, sterility of the preparation of the pig-derived transplant must be assured to avoid the transmission of infection to the recipient.

What is new since 2009: General aspects

Some new data have been developed since the previous version of the consensus statement published in 2009.

First, clinical studies transplanting pig islet cells have been performed and no transmission of PERV and other microorganisms has been observed [10-16]. Among these trials was the first New Zealand Government-approved clinical trial of alginate-encapsulated porcine islet cell transplants in fourteen patients suffering hypoglycemic unawareness. Each patient received between 5000 and 20,000 islet equivalents as a single dose from DPF Auckland Island strain

donor pigs. In advance of the trial, pigs and islet preparations were tested for 26 microorganisms (15 viruses, 10 bacterial species, and one protozoan) using molecular and immunological assays. Recipients were found to be negative on testing for PERVs and other microorganisms at multiple time points up to 1 yr following transplantation [16]. In addition, it has been reported that patients receiving viable pig skin demonstrate strong IgG responses to pig antigens but lack evidence of PERV infection up to 35 years post treatment. This is the longest time studied after xenotransplantation and shows that exposure to pig cells elicits a response, but more importantly, exposure evidently did not lead to infection [17].

Second, hepatitis E virus (HEV) and herpes viruses have been found in numerous animals even under SPF conditions using highly sensitive detection methods [6, 18-25]. The risk posed by HEV is difficult to evaluate. Only genotype (gt) 3 is associated with zoonotic transmission and severity of infection is dependent on a number of host factors [24]. There appears to be little clinical risk for healthy individuals; in some regions up to 56% of the adult population has been exposed to the virus as shown by detection of HEV-specific antibodies [19, 24]. There is great variation in the epidemiology of HEV and the risk posed to transplant recipients remains to be fully clarified in clinical studies. HEV gt 1 and 2 represents the greatest risk in pregnant women. In hyper-endemic gt1 and gt2 areas, pregnant women are at higher risk for severe disease and death, but this feature has not yet been reported for HEV gt3 infections. In pigs only gt3 and gt4 were found. HEV is also of risk for patients with underlying chronic liver conditions and immunosuppressed individuals, either by the human immunodeficiency virus (HIV) or by pharmaceutical immunosuppression in the context of transplantation [24-32]. Transmission of HEV via xenotransplantation has not been demonstrated and more studies are required to clarify any risks. It should be noted that the virus may be treated by the use of ribavirin based on studies of small numbers of immunosuppressed allograft recipients [33, 34]. Using newly developed highly sensitive methods, HEV gt3 was also detected in Göttingen Minipigs produced under spf conditions [8]. This may be explained by the finding that HEV can be transmitted from mothers to their piglets [8]. To improve the detection of PCMV also new detection methods were developed and used for screening [9].

What is new since 2009: PERV update

Although PERVs can infect (non-productively) cells of non-human primates (NHP) *in vitro* [35-38], transplantations of porcine tissues [39-42] and inoculations with highly concentrated

PERV preparations under immunosuppression [43] into NHP *in vivo* demonstrated no PERV transmission or infection, respectively. However, later investigations demonstrated that the major receptor for PERV-A is mutated in NHP and therefore the infection is not efficient [44]. This means that NHP do not represent a suitable model to be used for determination of the risk of transmission of PERV [45].

Sequencing of the pig genome [46, 47] and analysis of the prevalence [48-50] and expression [50] of PERVs in different pig breeds have shown the heterogenous nature of PERV distribution and differences between individual animals as well as breeds. With this in mind, simple screening for PERV loci cannot be applied routinely to all donor animals. However, this approach also provides an opportunity to select pigs with a lower expression of PERV-A and PERV-B if desired.

Improved methods allow better screening for PERV, both in the donor animals as well as in the human recipient (Table 1). Based on the fact that the human-tropic PERV-A, which is present in all pigs, can recombine with the ecotropic PERV-C, not present in all pigs, the selection of PERV-C-free animals may reduce the risk of PERV transmission to human recipients. Recombinant PERV-A/Cs are characterized by higher replication rates [51]. However, it is still unclear whether the exclusion of PERV-C positive animals to avoid recombination between PERV-A and PERV-C is important. There are no data that indicate any PERV infection in human recipients receiving donor islets from PERV-C positive animals [16].

Several restriction factors were characterized to be of particular importance for the replication of retroviruses: TRIM5 α , which disrupts the viral capsid after cell entry; TRIM28, which blocks viral transcription; ZAP (zinc-finger antiviral protein), which directs degradation of viral RNAs; tetherin, which traps virions on the surface of infected cells, and APOBEC (apolipoprotein B mRNA-editing catalytic polypeptides), which are cytidine deaminases that disrupt viral DNA during synthesis [52, 53]. Although PERV-A and PERV-A/C are insensitive to restriction by TRIM5 α molecules [54], overexpression of either human or porcine tetherin in pig cells significantly reduced PERV production [55]. In addition, human and porcine APOBEC3s could inhibit PERV replication [56-58], thereby reducing the risk of potential infection of human cells by PERV in the course of pig-to-human xenotransplantation. Further studies of antiviral restriction systems may help to develop therapeutic agents to regulate expression of these factors and to enhance antiviral activities.

To summarize, it is still unclear whether PERVs represent a risk in clinical xenotransplantation. No transmission of PERVs has been observed in multiple clinical trials enrolling more than 200 patients or up to 35 years post xenotransplantation [1, 10, 11, 16, 17]. However, most of the patients in the clinical trials were not exposed for a prolonged period to the xenotransplants and with some exceptions (associated with parallel kidney allotransplantation), no immunosuppression was applied. In addition, preclinical pig to non-human primate (NHP) transplantations, or infection experiments in small animals or NHP with or without pharmaceutical immunosuppression have not demonstrated infection [1, 37, 39-43]. It is meanwhile clear that NHP are not a suitable model to study the risk of PERV transmission since NHP carry – in contrast to humans – a mutated receptor for PERV allowing infection only with reduced affinity [44, 45]. Therefore, the question whether PERVs may be transmitted during xenotransplantation remains open. However, the availability of numerous sensitive and specific detection methods allows testing of the donor pigs and selection of suitable animals as well as screening of the xenotransplant recipients to detect a possible transmission very early. Indeed, selection of pigs free of PERV-C and with low expression of PERV-A and PERV-B is possible due to these excellent methods. Available antiretroviral agents have been shown to have activity against PERV in vitro [59-62]. Furthermore, genetic modification of donor pigs to exclude PERV loci, development of vaccines and other preventive strategies may be available in the near future. The potential viability of clinical xenotransplantation has resulted in continued investigation supported by the U.S. Public Health Service and the continued interest in the development of appropriate guidelines by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA). The first clinical trials of pig islet cell transplantation received regulatory approval in New Zealand [16] and in Argentina (Denner et al., in preparation) without reported adverse events. Strategies to reduce the expression of PERV by siRNA or to knock-out PERV by ZFN, TALENs, CRISPR/Cas technologies are under development. The present data indicate that - when using donor animals well characterized concerning PERVs and sensitive detection methods - PERVs are unlikely to provide a public health security risk in clinical xenotransplantation.

Most importantly, recent findings demonstrate that 62 genomic copies of PERV could be inactivated in an immortalised pig cell line by gene editing using CRISP/Cas9 [63]. This technology may be used in the future to derive porcine stem cells and embryos free of infectious endogenous retrovirus as well as to introduce desirable traits governing metabolic and immune functions. The impact of this advance remains to be explored [64]. Attempts to

inactivate PERV sequences in pig cells by gene editing using another nuclease, ZNF, failed [65].

Open questions 2016

The main question is: For which microorganisms should the recipient be monitored after xenotransplantation? The general consensus is that there is no need to monitor for pig-derived microorganisms absent in the donor pig. This assumes that available assays used in donor screening have the sensitivity required to avoid transmission of potential pathogens to immunosuppressed recipients. Assay validation might be examined in pre-clinical and clinical studies. This also requires the absence of infection during handling and transport. For animals free of known potential zoonotic pathogens, routine screening for PERV and, on the basis of clinical signs and symptoms, unknown pathogens, would be required. The methods to detect PERVs in the recipients are the same as used for pig screening (Table 1).

Potential infection by unknown or emerging microorganisms is interesting and remains a research endeavor. With new methods, e.g., next generation sequencing (NGS) including RNA sequencing, many new viruses or other microorganisms may be detected which are, as yet, of unknown clinical significance. For example, several novel astroviruses, bocaviruses and Ljungan-like viruses were identified in stool samples from healthy pigs in China, using high-throughput sequencing [66]. In a similar approach kobuviruses, rotaviruses, astroviruses, enteroviruses, sapoviruses, picobirnaviruses and a novel, previously unknown, virus, PigSCV, were detected in faeces of German pigs [67]. A new porcine parvovirus was recently described in U.S. pigs [68]. A long-term archiving of clinical specimens from donor swine and recipients was proposed; the optimal duration and modalities for such a repository remain to be described. The proficiency of the clinical laboratories charged with testing donor and recipient samples is essential to assure both researchers and the public regarding the stringency of clinical safeguards. This may require advanced, accredited (e.g., GLP) laboratories available in major academic centers or Contract Research Organizations (CROs) and needs authorization by the competent regulatory authorities. Such laboratories must have the capacity also to test samples for human organisms that may infect transplanted porcine cells and tissues. Many recipients will have prior serological and clinical data available to indicate prior exposures to latent or persistent organisms such as the herpesviruses, hepatitis B or C viruses, HIV, or HEV. It is not known whether such pathogens will infect islets or encapsulated islets – such studies are required if the organism has the capacity to infect

porcine cells *in vitro* or *in vivo*. The infectious challenge posed by encapsulated cells and tissues in non-immunosuppressed recipients may be less than that in immunosuppressed recipients of cellular or vascularized xenotransplants. Additional information may be obtained through use of standardized WHO questionnaires for recipients to indicate any changes in health status and the use of the ‘precautionary principle’ [69]. That is to be prepared in advance for the identification, evaluation, and response to infectious syndromes. The monitoring of close contacts of the recipients should not be required unless data exist to demonstrate that the recipient is infected. It is not generally considered that transmissible spongiform encephalitis is a likely concern for islet xenotransplantation and is not a consideration of current WHO pathogen lists since there are no indications for prions in pigs. In contrast, prion transmission has been discussed in the context of islet allotransplantation [70].

Emerging viral concerns

As mentioned above, it appears that the potential for emerging viruses from donor or recipient would be of concern in the absence of other potential zoonotic pathogens [5]. In islet cell allotransplantation, a number of transmissions have been documented, the most common pathogens being CMV and enteroviruses; other viruses including HIV-1, HCV, lymphocytic choriomeningitis virus (LCMV), and rabies virus have been transmitted from organ donors to recipients [71-78]. To date no emerging viral disease, as has been seen for human solid organ transplantation [75] has been documented in islet cell allo- or xenotransplantation [5]. In this context the zoonotic potential of arenaviruses has been discussed [79]. Recognition of novel infections in immunosuppressed hosts can be difficult as the manifestations of infection including inflammation may be absent. Given that encapsulation of islets may reduce or negate the need for clinical immunosuppression of the recipient, the likelihood of infection may be reduced and any organ-derived infection may be more clearly recognized. As discussed above, routinely applied NGS or RNA-sequencing could potentially identify novel/unknown pathogens to provide a microbiologic diagnosis.

With regard to PERV, as already reported in the consensus statement of 2009, different strategies have been developed to increase viral safety largely by preventing transmission of PERV. These strategies include vaccine development [80-84], RNA interference to knock down the PERV expression [85-87] and directed nuclease (e.g. ZFN, TALENs,

CRISPR/Cas9)-based knock out of PERV [63-65, 88, 89]. However, due to lack of PERV transmission the value of these techniques in a clinical setting has yet to be evaluated.

The clinical application of gene editing technology to the enhancement of xenotransplant safety is presently unknown. Other approaches to donor genetic modification (e.g., breeding) and to the reduction in infectious risk (e.g., monitoring, encapsulation) may also serve to enhance clinical safety and are under investigation.

Table: Methods to be used to detect microorganisms in the donor pig and if necessary in the recipient

Method	What can be detected
Direct detection methods	
PCR, real-time PCR	DNA microorganisms
RT-PCR, real-time RT-PCR	RNA microorganisms, gene expression
Immunofluorescence, , Immunohistochemistry,	Protein expression
Western blot analysis	
Electron microscopy	Microorganisms
Indirect detection methods	
ELISA, Western blot analysis	Detection of antibodies

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