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Review

XENOZOONOSIS IN XENOTRANSPLANTATION: CLINICAL CHALLENGES

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Summary

Preclinical and clinical xenotransplantation trials performed until now have clearly demonstrated that the outcomes depend significantly on the prevention of xenozoonoses. In some preclinical trials where pig kidneys and hearts were transplanted into different non-human primates (marmosets, baboons, rhesus and cynomolgus monkeys) transmission of pig viruses was observed. Specifically, porcine cytomegalovirus (PCVM), a porcine roseolovirus (PRV), was transmitted causing a significant reduction of the survival time of the transplant. The official name of the virus is suid herpesvirus 2 (SuHV-2). Furthermore, porcine circovirus type 3 (PCV3) was also transmitted in preclinical trials. In this case no clinical signs were observed. In over 200 human patients receiving different pig materials including islet cells, nerve cells, skin as well as in *ex vivo* perfusions of pig organs, no porcine endogenous retroviruses (PERVs), which are integrated in the genome of all pigs, could establish itself in the new host. However, the presence of other viruses had not been analyzed and the survival time of the transplants was short. In clinical trials encapsulated pig islet cells from Auckland Island donor pigs were transplanted into diabetic humans in New Zealand and Argentina. The donor animals were microbiologically clean and well characterized, 14 viruses were found absent in the Auckland Island pigs and none of these viruses including PERV were transmitted to the recipients. In the first transplantation of a heart from a cloned genetically modified pig into a patient in Baltimore, PCMV/PRV was transmitted, contributing among other factors to the death of patient. The results underline that donor animals must be well characterized using sensitive and specific detection systems for porcine viruses.

Key words: xenotransplantation, preclinical trials, clinical trials, porcine viruses, non-human primates, porcine cytomegalovirus/porcine roseolovirus (PCVM/PRV), porcine endogenous retroviruses (PERVs)

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INTRODUCTION

Xenotransplantation has the unique potential to transmit pathogens from a donor animal graft to a recipient [1]. Using specialized breeding techniques, most of the potential pathogens can be removed from the donor animal [2]. In particular, bacterial and fungal pathogens (and most of the viruses) are acquired during or after birth, and infection can be avoided by preventing

contact with infected animals, resulting in a designated pathogen free status [1]. Two specific features inherent to some viruses pose the main challenge to this strategy: Porcine endogenous retroviruses have a genomic origin and are passed on to any offspring. Inactivation is potentially possible by genetic manipulation but remains an ambitious goal [3,4]. Most latent viruses on the other hand are avoidable by early weaning, e.g. growing up of the piglets without maternal milk [5]. Proof of absence is however demanding as direct detection methods including very sensitive PCR methods may yield negative results despite ongoing latency. Our article will focus on the most challenging viral pathogens and summarize the current knowledge.

TRANSMISSION OF PORCINE VIRUSES IN PRE-CLINICAL LARGE ANIMAL TRIALS

The potential of PERVs to infect human cells *in vitro* has been one of the main concerns in xenotransplantation. Intense efforts to look for infection in pre-clinical trials were undertaken, with the main challenge to discern microchimerism and true infection of human cells [6-10]. The mere detection of PERV DNA does not allow to prove active infection. Integration of PERV into the human genome has to be demonstrated using specific methods [10]. A positive serology would at least indicate an active immune response of the recipient, possibly the result of an active infection. However, a reliable serology is notoriously difficult to develop, and sensitivity and specificity are often difficult to establish [11]. All studies in pre-clinical models have so far failed to establish active infection of PERV. In some recipients, PERV DNA was indeed found in the blood and organs, but in conjunction with donor pig DNA, pointing to microchimerism rather than active infection [7,9]. Serology, when used, remained negative [9,12-14].

Accordingly, similar issues arise with PCMV/PRV. *In vitro,* there is no conclusive evidence that PCMV/PRV infects human cells [15]. In line, in recipients of a xenotransplant, detection of PCMV DNA outside of the transplant was accompanied by the detection of donor DNA, again indicating microchimerism [16]. Even if active cross-species infection was not observed, transmission of PCMV/PRV into the recipient was associated with deleterious consequences [2,9,17]. High copy numbers of PCMV/PRV DNAemia were detected in transplanted porcine transplant tissues [17]. High virus loads were also detected in most organs of a baboon recipient and, by immunohistochemistry, virus protein expressed in cells have been demonstrated in nearly all baboon tissues [9,16]. The association of PCMV/PRV and a consumptive coagulopathy observed in pig-to baboon kidney xenotransplantation was indirectly suggested by its absence in PCMV/PRV free donor [2]. An increase of porcine tissue factor in an *in vitro* model of primary porcine aortic endothelial cells infected with PCMV/PRV potentially provided an important link of the coagulopathy to PCMV/PRV [18]. Early weaning of piglets led to an improved graft survival, presumably by elimination of PCMV/PRV [5]. The role of PCMV/PRV was corroborated by a recently published study in an orthotopic heart transplantation model [9]. In short, baboon recipients of a PCMV/PRV-positive donor heart, developed elevated IL-6, TNF- and tPA-PAI-1 complex levels pointing to an interference with the cytokine and coagulation system.

TRANSMISSION OF PORCINE VIRUSES IN HUMAN XENOTRANSPLANTATION

Until now more than 200 human individuals received pig tissues to treat diseases such as diabetes (islet cells), hemophilia (porcine factor VIII), treatment of burns (pig skin), and neurological diseases (neuronal cells) or were connected to bioartificial liver devices for the treatment of liver failure, extracorporeal kidney perfusion for the treatment of kidney disease, and extracorporeal spleen perfusion (for review see [19]). The number of pig cells transplanted to the recipients was low (for example 400 million to 2 billion uncapsulated pig islet cells) [20] and the duration of *ex vivo* perfusion was short (50 minutes to 4.25 hours) [21], and there was no pharmaceutical immunosuppression applied. In none of these transplantations or extracorporeal perfusions porcine endogenous retroviruses (PERVs) had been transmitted. The transmission of other porcine viruses was studied only in two trials where encapsulated islet cells from Auckland Island pigs were transplanted [22-24]. Based on their history, the Auckland Island pigs were characterized by the absence of many porcine viruses. Porcine circoviruses 1 and 2 (PCV1, PCV2), porcine lymphotropic herpesvirus-2 (PLHV-2), porcine cytomegalovirus (PCMV/PRV), rotavirus (RV), porcine teschovirus (PTV), porcine parvovirus (PPV), encephalomyocarditis virus (EMCV), bovine viral diarrhea virus (BVDV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine enterovirus type 1 and 2 (PEV1, 2), pseudorabies virus (PrV) or suid herpesvirus 1 (SuHV-1) and hepatitis E virus (HEV) were absent in these animals. And therefore, none of these viruses were transmitted in clinical trials in New Zealand and Argentina using encapsulated islet cells from Auckland Island pigs. Furthermore, PERV was also not transmitted.

The first transplantation of a large vascularized organ under severe immunosuppression into a living patient took place in January 2022: a heart from a genetically modified

pig was transplanted into a 57 years old patient by a group at University of Maryland in Baltimore (UMD) [25]. The donor animal had 10 genetic modifications, including targeted insertion of two human complement inhibitor genes (decay-accelerating factor, membrane cofactor protein CD46), two human anticoagulant genes (thrombomodulin; endothelial cell protein C receptor), and two human immunomodulatory genes (integrin associated protein CD47, heme oxygenase), as well as deletion (knockout, KO) of three pig carbohydrate antigens (galactose- 1,3-galactose, Gal; N-glycolylneuraminic acid, Neu5Gc; and the Sda blood group carbohydrate being the product of the enzyme, -1, 4 -N-acetylgalactosaminyltransferase) and the pig growth hormone receptor gene. The donor pig did not express red blood cell antigens and was therefore a universal donor with respect to blood type. The donor pig originated from a group of similarly bred pigs from Revivicor, Inc., a subsidiary of United Therapeutics Corporation. While the donor pig was tested for PCMV/PRV, the test was only done using a single detection method on a single sample type collected only once (a PCR of a nasal swab was performed). PCMV/PRV infection is however only detectable by PCR in recently infected pigs. Hence, PCMV/PRV in the donor pig was missed and the virus was transmitted to the patient and contributed among other factors to the death of the patient [25]. The virus load steadily increased in the blood of the patient because the virus replicated unrestricted in the donor heart due to the absence of the primed pig immune system. The clinical signs in the Baltimore patient resembled the signs in baboons after an orthotopic transplantation of a PCMV/PRV-positive pig heart: cytokine release, problems with coagulation and multi organ failure [9]. Since there is no evidence that PCMV/PRV infects human cells [15,26], it is assumed that the virus interacts with the immune cells and endothelial cells and induced so the clinical signs.

The first two pig kidney transplants after bilateral nephrectomy were performed at the University of Alabama in Birmingham (UAB) into a brain-death 57-year-old male in 2021 [27]. Here also a Revivicor pig with 10 genetic modifications was used. There was some renal function after transplantation, but thrombotic angiopathy, acute tubular necrosis, endothelial cell swelling with some thrombosis were observed. The experiment was terminated after 74 hours, a time too short to suggest transmission of porcine viruses. Nevertheless, the signs mentioned above may be the result of PCMV activity [28].

Also in 2021, the transplant group at New York University (NYU) Langone Transplant Institute conducted kidney transplants using two brain-death individuals, with the kidneys kept outside the body [29]. The donor animals were genetically modified alpha-1,3-galactosyltransferase–knockout (GalT-KO) pigs again originating from Revivicor. The kidneys functioned and produced urine for 54 hours at which time the experiment was terminated, a time too short to prove transmission of porcine viruses.

PREVENTION OF TRANSMISSION OF EXOGENOUS AND ENDOGENOUS VIRUSES

The pig virome includes numerous single and double stranded DNA and RNA viruses such as member of the families of the *Adenoviridae, Anelloviridae, Astroviridae, Caliciviridae, Circoviridae, Parvoviridae, Reoviridae, Picornaviridae* and others as detected by next generation sequencing [30]. These listed virus families contain the most common viruses in healthy animals. At the moment it is difficult to judge which viruses have the potential to be transmitted to a human recipient, which viruses are able to infect human cells and which viruses may be capable to induce a zoonotic infection. Furthermore, it seems that viruses with a potentially greater risk of becoming zoonotic are present in the pigs at a low virus load and therefore detectable only with highly sensitive PCR methods. The main DNA viruses significantly infecting pig livestock are porcine circoviruses (PCVs), African swine fever virus (ASFV), porcine parvoviruses (PPV), and pseudorabies virus (PrV) [31]. PCVs and PPV are small viruses having one capsid protein and short genomic single-stranded DNA. Vaccines against both viruses have been developed. It is necessary to design new vaccines against PCV and PPV since new genetically different genotypes yet of unknown pathogenicity have been demonstrated. In contrast, the ASFV and PrV are large viruses composed of a trilayer envelope and long linear genomic dsDNA. In the case of the ASFV, there are many approaches to vaccine development. However, at this point the effectiveness of previously produced preparations may not be sufficient and the overall market may be too small for commercial purposes, e.g., there is no commercial vaccine. In the case of the PRV, the majority of developed vaccines are live attenuated vaccines which in general work well. Swine influenza is a highly contagious viral infection of pigs caused by the swine influenza virus (SIV) [32]. Swine flu only infects a few individual people each year with close contact to pigs (fairs) and has not been shown to transfer from infected humans to other humans. However, recombinant H1N1 spread quickly in the human population and became a pandemic, causing 60 million cases, and 12,500 deaths in the United States (https://www.cdc.gov/flu/pandemic-resources/2009-h1n1-pandemic.html).

Hepatitis E is caused by an RNA virus, hepatitis E virus (HEV). In pigs HEV genotype 3 is most prevalent (HEVgt3 or HEV-3). HEV-3 is usually transmitted by consuming under[1]cooked pork or environmental contamination

with pig manure. In people, it can cause liver infections, in addition to chronic infection and neurological symptoms in immunocompromised individuals [33]. Rabies is a deadly virus caused by the rabies virus (RV). Rabies is rarely transmitted from pigs. Symptoms include fever, headache, confusion and abnormal behavior and death is almost certain.

Simple methods are available to detect porcine viruses and to prevent transmission of exogenous viruses. A comprehensive list of publications describing detection methods for several viruses such as PCMV/PRV, HEV, PLHV-1, PLHV-2, PLHV-3, PCV1, PCV2, PCV3, PCV4, PPV1, TTSuV1, TTSuV2, and SARS-CoV-2 is given in [34]. Whereas most viruses can be detected by PCR-based methods, latent viruses such as PCMV/PRV require additional testing using immunological methods [35]. In both cases complex detection systems have been established, which include in addition to the mentioned PCR-based and immunological detection methods also cell-based methods such as infectivity assays, the time of the sample generation, the origin of the sample, the preparation of the sample as well as positive and negative controls [34]. Furthermore, some viruses can also be transmitted via oocytes, which are subsequently used for microinjection or somatic cell nuclear transfer (SCNT) [36].

In addition to the detection methods there are excellent elimination strategies [34]. These elimination strategies require highly sensitive detection methods (including PCR assays as mentioned above) to confirm that virus elimination was indeed successful. If no potentially zoonotic viruses are being found in a given pig, this pig can be used directly for xenotransplantation. On the other hand, if a confirmed infection of the pig is characterized by a high virus load of a particular virus, the pig cannot be used and should be eliminated. In the case of a low virus load, the virus can be eliminated by passive vaccination, treatment with antiviral drugs, or early weaning, caesarean delivery, colostrum deprivation, or embryo transfer. To limit any possible de novo virus introduction and replication in a xenotransplantation donor herd perhaps a prophylactic xenotransplantation vaccination regimen could be put int place. In some cases, however, vaccination may hinder the detection of infection and should be avoided. It is important that the animals are repeatedly tested and kept in isolation to avoid that viruses infect them *de novo*.

Porcine endogenous retroviruses (PERVs) are integrated in the genome of all pigs and cannot be eliminated this way. Until now successful transmission of PERV in preclinical trials has not been demonstrated in non-human primates (partially due to the fact that the PERV receptor in non-human primates do not allow massive virus replication). An absence of PERV transmission was observed in the first clinical transplantations of encapsulated pig islet cells in New Zealand and Argentina (partially due to the encapsulation and low numbers of cells that were transferred). PERV was also not transmitted in infection experiments inoculating high amounts of high-titer PERV into non-human primates [37].

There are up to 60 copies of PERV in the pig genome, PERV-A and PERV-B are present in all pigs and are able to infect human cells, whereas PERV-C is present in most but not all pigs. The integrated proviruses are still active and replicate and integrated *de novo* so that the number of proviruses usually differs in different organs (for review see [38]). Furthermore, it has been demonstrated that PERV-A and PERV-C can recombine and the resulting PERV-A/C recombinant is capable to infect human cells and the generated infection is characterized by higher replication rates compared with the paternal PERV-A. Since PERVs are integrated in the pig genome, they cannot be eliminated by the strategies mentioned above for exogenous viruses. Although until now transmission of PERVs in preclinical and clinical trials has not been observed [39], several strategies have been developed to prevent PERV transmission, including selection of PERV-C -negative pigs in order to prevent recombination between PERV-A and PERV-C, vaccination [40-42], antiretroviral drugs (for review see [43]), siRNA suppressing expression of PERV [44,45], and inactivation by CRISPR/Cas9 [3,4].

POST XENOTRANSPLANTATION SCREENING OF PATIENTS

In the last years numerous PCR-based and immunological assays have been developed to screen donor pigs for potentially zoonotic or xenozoonotic microorganisms. The same methods are being used to follow the non-human primate recipients in the case of preclinical trials or the patients in the case of clinical trials. As a matter of fact, theoretically there is no need to test the recipients for microorganisms which were absent in the donor pig. However, in the case that viruses or other microorganisms were present in the donor pig at very low quantities and below the detection limit of the diagnostic assays used, additional screening of the patient at one or several time points after the transplantation may be advisable. For some specific viruses such as PERVs which cannot be eliminated easily from the donor pig, besides perhaps usage of CRSPR/Cas, a repeated testing may be advised. If the patient is not infected there is no need to test contact persons or clinical personal and there is no need to forbid sex.

There are several questions which are difficult to answer at the moment and which have to be discussed after future progress in xenotransplantation. First, how likely will it be that PERVs will be activated years after

the xenotransplantation, infect and adapt to human cells as described in *in vitro* adaptation experiments [46]? Second, how long do reference tissues from the donor pig have to be stored for scientific studies after the transplantation has been conducted? To answer the last question, the situation in allotransplantation has to be analysed.

CONCLUSIONS: RECOMMENDATIONS FOR CLINICAL TRIALS

Using the highly sensitive virus genome detection systems and elimination strategies it should be possible to perform future xenotransplantations using pigs free of potentially zoonotic viruses [47]. However, in rare cases and despite all efforts to screen donor pigs, transmission of known or unknown pathogens cannot be excluded. Regular follow-up of recipients must be ensured, as must procedures be in place for work-up of patients with a presumed infection. Diagnostic tools for the common potentially zoonotic porcine microorganisms, in particular for the viruses discussed above, should be available in transplantation centers.

Protocols for regular sampling and surveillance have been proposed [1,19,48]. Of special relevance is the availability of stored samples drawn at predefined intervals regardless of clinical symptoms. In patients with signs of infection, a diligent work-up must be performed before starting empirical therapy. Infection control measures should be applied taking into account presentation, and maintained until diagnosis has been established [1]. Ideally, hypothesis-free diagnostic options, such as metagenomics next generation sequencing, and broad range PCR should be accessible in a timely fashion. Careful preparation of the entire team involved is paramount, also to alleviate potential fears. These measures must be maintained, but hopefully will not be applied, because xenotransplantation is safe if done properly.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

NJM, TO, JD: all wrote and critically revised the manuscript.

Ethical consideration

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