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Infection in Xenotransplantation: Opportunities and challenges

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Abstract

Purpose—Post-transplantation infections are common. It is anticipated that infection will be no less common in xenotransplantation recipients. Prolonged xenograft survivals have resulted from advances in immunosuppressive strategies and development of swine that decrease host immune responses via genetic manipulation, notably CRISPR/cas9 manipulation. As prospects for clinical trials improve, consideration of the unique infectious risks posed by xenotransplantation reemerge.

Recent Findings—Organisms likely to cause infection in human recipients of porcine xenografts are unknown in advance of clinical trials. Microbiological screening of swine intended as xenograft donors can be more intensive than is currently feasible for human allograft donors. Monitoring infection in recipients will also be more intensive. Key opportunities in infectious diseases of xenotransplantation include major technological advances in evaluation of the microbiome by unbiased metagenomic sequencing, assessments of some risks posed by porcine endogenous retroviruses (PERVs) including antiretroviral susceptibilities, availability of swine with deletion of genomic PERVs, and recognition of the rapidly changing epidemiology of infection in swine worldwide.

Summary—Unknown infectious risks in xenotransplantation requires application of advanced microbiological techniques to discern and prevent infection in graft recipients. Clinical trials will provide an opportunity to advance the safety of all of organ transplantation.

Keywords

Xenotransplantation; xenosis; porcine endogenous retrovirus (PERV); metagenomic sequencing; microbiome; donor-derived infection; CRISPR/cas9; gene editing

Introduction

Immunosuppression for xenotransplantation of porcine organs in primates and clinical trials carries a risk for opportunistic infection proportional to the intensity of immune deficits and epidemiological exposures of recipients. The unique challenges posed by transplantation of organs between species are the limited data on the microbiology of swine – specifically which organisms are likely pathogens for humans. Experience with pig-to-nonhuman primate transplantation provides insights into potential microbes that may cause infection in

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recipients. This allows development of programs for screening of source animals and organs for potential human pathogens, studies of the biology of these organisms, and technology to monitor recipients for infection. As for any immunocompromised individual with infection, microbiologic identification of specific organisms causing infection allows targeted therapy while avoiding side effects of unnecessary antimicrobials. Advances in microbiology including quantitative molecular assays for viruses and unbiased metagenomic sequencing techniques, allow recipients and donors to be screened and monitored for infections even when asymptomatic. Pig-specific pathogens such as porcine endogenous retroviruses (PERVs) or circoviruses may be excluded from swine colonies and monitored in recipients. The application of newer techniques will supplement pathogen-specific assays including broad-range molecular probes, microarrays and high throughput pyrosequencing. Unique challenges are presented by the array of pig-specific organisms for which diagnostic tools are needed and changing worldwide epidemiology of infection in swine.

Xenotransplantation allows development of approaches that will increase microbiological safety in transplantation while improving the supply of transplantable organs.

Introduction

Infectious Challenges in Xenotransplantation

Following organ transplantation, infection and cancer are the main complications of long-term immunosuppression. Infection comes from the environment, from the recipients themselves (i.e., prior colonization or latent and active infection), and by microbes traveling with the transplanted organ[1–3]. The overall risk for infection is determined by host factors (technical skill, immunogenetics, preventative strategies) coupled with the impact of the immunosuppression applied -- the "*net state of immunosuppression"*. The net state of immunosuppression includes all the factors contributing to infectious risk in an individual – a summation of the immunosuppression, metabolic derangements, viral coinfections infection, and technical complications. If infectious risk can be reduced, more intensive immunosuppression can be applied safely.

The unique challenge of xenotransplantation is the relative paucity of data regarding the microbiology of normal and genetically modified swine, despite the commercial importance of the species. The microbiological behavior of zoonoses in the immunosuppressed human host cannot be predicted. In the absence of clinical trials, the "infectious risks" of xenotransplantation are guesses based on extensive experience with immunosuppressed human allograft recipients and in studies of immunosuppressed swine and primate xenograft recipients. Based on these data, organisms thought likely to cause infection in human recipients can be minimized via screening and exclusion of potential pathogens during animal rearing (Table 1)[4]. The mechanisms applied for such exclusion (vaccination, antimicrobial treatments, animal isolation) can vary. Xenograft recipients will undergo prospective assessments for known organisms and, in with infectious syndromes (which are inevitable), by intensive investigation, empiric therapies, and creative application of newer technologies for microbial detection (discussed below).

The term "xenosis" (also "direct zoonosis" or "xenozoonosis") reflects the unique epidemiology of infection due to organisms from a nonhuman source species transmitted

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with xenogeneic grafts[4–6]. Intensive investigation revealed some previously uncharacterized species including the porcine endogenous retroviruses or PERVs[7–9]. PERVs are nonpathogenic in swine; human cells carry PERV receptors. Experience with retroviral transmission between species suggest such genomic elements, if expressed, have the potential to cause disease in another species ("xenotropic organisms") or acquire new characteristics via genetic recombination or mutation including increased virulence[10–15]. Infection may persist within grafts due to the incompatibility of histocompatibility antigens between species which reduces the efficacy of immune responses. Based on clinical experience, some newer organisms recently described in swine might be considered including porcine polyomaviruses, coronaviruses, and Borrelia species – which have never been detected in humans[16–19]. In addition, epidemics of African Swine Fever virus have spread throughout Asia recently but are of unlikely clinical importance other than inhibiting the exchange of pigs between affected and unaffected regions[20].

Epidemiology

Identification of new organisms in swine may provoke anxiety in investigators and regulatory authorities for clinical trials (as for PERVs). However, d*evelopment of sensitive and specific microbiological assays for use in breeding, donor and organ screening, monitoring or diagnosis becomes feasible only when such potential pathogens are discovered.* In addition to (e.g.,) quantitative molecular assays for each organism, serological tests or measures of T-lymphocyte immunity (e.g., pathogen-specific interferon-gamma release assays) are useful in screening populations to identify prior exposures and latent infections (Table 1). These require validation both in swine and human xenograft recipients as assays perform differently in human and porcine sera. It is notable that most assays for animal-derived organisms in humans are unavailable with exceptions being common pathogens such as toxoplasmosis or influenza. Such assays may be limited to commercial or veterinary laboratories. Serologic tests are often falsely negative in the immunocompromised host.

Metagenomic sequencing

Rather than microbiology driven by each "organism du jour," new types of assays merit consideration. Agnostic metagenomic "next generation sequencing" (mNGS) are universal pathogen detection methods for microbiology [21]. These methods provide genomic characterization of all types of organisms without bias based on clinical syndrome (often absent) or limitation to pathogen-specific assays[22]. This might be considered "hypothesis-free testing"[22]. Current techniques are limited by requirements for comparator organism sequence data from both swine and humans, method standardization, the challenge of differentiating colonization from invasive infection, risks for specimen contamination by host and environmental nucleic acids, and data interpretation[21,23]. Metagenomic approaches to the determination of transmission of zoonotic microorganisms is feasible using blood and tissue samples or cell free nucleic acids released during the course of infection (Table 1) [24]. These techniques are particularly useful in asymptomatic individuals (e.g., immunocompromised hosts or for monitoring) or for detection of previously unrecognized pathogens [23]. It is possible that microbiome analysis of donor species could identify potential pathogens in advance of clinical trials[25]. Microbiome studies determine the

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composition and function of a community of microorganisms in an anatomic site; the pathophysiological importance of such determinations in transplantation are under investigation[25,26].

Source Animal Development

"Exclusion lists" of organisms thought to pose unacceptable risks to xenograft recipients were developed as a basis for testing in breeding colonies ("Designated Pathogen Free Colonies", Table 2)[4]. As was noted, it should not matter how such exclusion is achieved if the designated organisms are demonstrably absent from the source herd, animal, or organ. Such lists must be dynamic based on changing epidemiology and data from experimental and clinical experience[4,27,28]. Microbiological assessments can be made for sentinel animals and specific animals selected for organ procurement. Pig health is governed by standard veterinary practice including vaccinations, microbially-restricted diets, filtered water, avoidance of unnecessary antibiotics, and "biosecure facilities" to prevent introduction of microbes from rodents, insects, and birds. A "Designated Pathogen-Free Exclusion List" was developed for organisms like those associated with human allotransplantation (Table 2). Of these, only hepatitis E virus (HEV) has been identified in immunosuppressed humans. Regulatory guidance documents exist for clinical trials[29–33]. These provide an outline of essential considerations for clinical trials including infectious disease management[31]. Such trials must be performed in transplantation centers with laboratory expertise to identify potential donor-derived pathogens. Guidance notes that there needs to be the capacity to test for latent viruses or pathogens, which may require development and validation of new assays[31]. As was noted, the availability of multiple overlapping diagnostic tests and agnostic assays such a mNGS would be advantageous.

The Impact of Viral Infection after Xenotransplantation

Viral infections are common after organ transplantation given the efficient transmission of viruses with living cells coupled with intensive immunosuppression. Diagnosis of porcine viral infections has been addressed, in part, by development of sensitive, quantitative molecular assays for PERVs, porcine lymphotropic herpesvirus (PLHV), porcine cytomegalovirus (PCMV), circoviruses, and adenoviruses[4]. Infection by these viruses in humans has not been reported; each is associated with a specific clinical syndrome in swine and in nonhuman primate xenograft recipients. PCMV infection is restricted to porcine tissues causing endothelial activation, consumptive coagulopathy and early graft loss [34–37]. PCMV can be excluded from pig colonies by early weaning and isolation but is easily reintroduced into herds. PLHV is associated with a form of post-transplantation lymphoproliferative disorder (PTLD) in immunosuppressed swine undergoing stem cell transplantation but causes no known disease in primates [38,39]. Porcine circovirus type 2 (PCV2) causes pneumonia and wasting syndrome and immune dysfunction but no known infection in primates [40,41].

Pig genomes contain diverse endogenous beta- and gamma-retroviruses, most of which appear to be replication defective. As noted by Robin Weiss, these endogenous elements, even if incomplete, might contribute via recombination or reinsertion events to the development of novel genomic PERV strains[15]. The successful removal of barriers to

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hyperacute graft rejection (knockout of alpha-1,3-galactosyltransferase genes and the insertion of genes for human complement regulatory proteins) may also remove barriers to infection by enveloped viruses[15]. In swine, our isolation and sequencing of PERV was based on early studies of a retrovirus associated with swine lymphoma (reviewed in [4])[7,42–50]. Three related C-type porcine endogenous retroviruses (PERV A, B, C) have been identified in swine that possess infectious potential. Human cellular receptors for PERV-A, HuPAR-1 and HuPAR-2, have been identified[51]. PERV-A and -B, can infect human and pig cells *in vitro,* while PERV-C infects only pig cells[7,46,48,51–57]. Exogenous recombinant viruses containing the receptor-binding site of PERV-A and segments of PERV-C (PERV AC) have high replication efficiency in vitro; we showed that these may cause autoinfection with reintegration as genomic AC recombinants[55,58–60].

While PERV mRNAs are expressed in all pig tissues and in all breeds of swine tested to date; genomic PERV types and the level of expression vary and some swine lack PERV-C or a recombination locus [7,61–66]. Lung, spleen, and lymph node consistently show high levels of PERV expression, possibly reflecting leukocyte content. While PERV expression is lower in whole pancreas, PERV expression is equally high in isolated pancreatic islets[67]. Relative to detection strategies, in vitro culture of islets did not reveal reverse transcriptase or PERV virus, suggesting limitations to current detection strategies[67]. Induction of porcine pluripotent stem cells (piPSCs) produces 10-fold to 100-fold higher transcription of the viral PERV-A and PERV-B envelope genes (env), viral protease/polymerase, and L1 elements without detection of functional retrovirus[68]. While enhancement of viral gene expression by viral and cellular factors acting in trans has been demonstrated between human herpesviruses and endogenous (HERV) and exogenous (HIV) retroviruses, PCMV coininfection does not alter the replication of PERV in life-supporting renal xenotransplantation in vivo in baboons[69].

Despite the presence of functional receptors on human cells, preclinical and clinical xenotransplantation studies using pig cells, tissues, and organs have failed to demonstrate transmission of PERV to humans in vivo and to most normal human cells in vitro. This suggests either inadequate exposure to human-tropic, replication competent virus, or protection by intrinsic cellular antiviral mechanisms. PERV does not replicate well in non-human primate cells making studies in primates less informative [70,71]. Should infection occur, PERV is susceptible in vitro to clinically available nucleoside and non-nucleoside reverse transcriptase inhibitors [56,72–77]. In in vitro studies, adefovir demonstrated moderate inhibition of PERV replication while nevirapine has more limited PERV inhibitory activity; integrase inhibitors including raltegravir, dolutegravir and inhibited PERV replication at the nanomolar levels[78]. Interestingly, riboflavin, the natural ligand for PERV-A receptors on human cells (SCL52A, a riboflavin transporter) produced no inhibition of PERV infection suggesting that alternative entry mechanisms may exist[78].

Theoretical strategies to prevent PERV transmission include the use of PERV-C-negative or low virus producing pigs, vaccination, antiretroviral therapy, RNA interference therapies and creation of PERV knockout animals using CRISPR-Cas9 or other gene editing techniques[79–81]. Using CRISPR-Cas9 to target the polymerase gene of PERV elements, inactivation was achieved of all 62 copies of PERV in the immortalized porcine kidney

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epithelial cell line PK15 which normally releases high levels of infectious PERV in vitro[82]. All PERV elements were mutated; viral replication and reverse transcriptase activity were not detected. Transmission to human cells in coculture studies was no longer demonstrable[82]. PERV C-negative pig fibroblasts were similarly treated for use in somatic cell nuclear transfer to generate PERV-inactivated embryos, carried by PERV-C negative surrogate sows[81]. While the infectivity status of these fibroblasts at baseline was not presented, PERV inactivation (~25 copies) was confirmed at the DNA and RNA levels; further studies are underway. Xenografts from these animals have not yet been reported. Concerns regarding off-site genomic modifications by CRISPR technology are under investigation [83]. Pigs with PERV deletions require investigation for unanticipated genetic and physiologic changes.

Towards Clinical Trials of Xenotransplantation

Early clinical trials will inform appropriate monitoring and prophylaxis strategies for xenotransplantation. Infections should be anticipated in any group of immunosuppressed organ recipients – some early when immunosuppression is most intense and technical complications are most common and lifelong as graft function may vary[4]. Routine pretransplant screening of recipients will recognize some latent infections that merit surveillance or prophylactic therapies; these include tuberculosis, CMV, EBV, and hepatitis B or C viruses. Donor-derived infections can be limited by the screening of source animals to the extent of available assays for latent as well as active infections. This suggest a utility for serologic testing and development of T-cell assays to detect immune memory for relevant (e.g., viral) organisms. Routine monitoring for known and unknown organisms, as per FDA and other guidance documents, will apply microbe-specific assays (cultures, quantitative molecular assays as outlined in Table 3) and can also begin to apply some advanced metagenomic sequencing methods to surveillance[31]. These are not yet validated or approved for clinical use, are both costly and not optimized for use in the combined human-porcine nucleic acid environment[21–24]. Samples from recipients, and possibly from close social or sexual contacts, may be archived at standard timepoints against future epidemiologic studies or improvements in unbiased metagenomic sequencing. These could include blood samples to assess (e.g.,) peripheral blood chimerism for pig cells. Blood and tissue (biopsy) samples will be aliquoted and stored at multiple sites in appropriate storage media for RNA, DNA, cell and serum proteins. Routine nucleic acid testing can be performed for PERV (A, B, C, AC), PLHV and PCMV (if present in donor), and for common human viruses (human CMV, adenovirus, EBV). If PERV is present in donor swine, cocultivation of peripheral blood leukocytes with virus-permissive human and porcine cell lines may be informative.

Organ transplant recipients frequently develop signs of infection such as fever, gastrointestinal, urinary tract or respiratory symptoms, unexplained leukocytosis, hypotension, graft dysfunction, or abnormal metabolic testing. At such times, surveillance studies can be repeated. Clinical evaluations will be largely syndrome-driven including blood, urine and/or sputum cultures and appropriate radiographic testing and invasive diagnostic procedures for microbiology and histopathology. Empiric antimicrobial therapy can then be initiated.

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Initial xenograft recipients should be screened for latent infections and surveillance and prophylactic strategies developed. These need not be different than those for allograft recipients. Ideally, early recipients should not be colonized with antimicrobial-resistant organisms. Protocols including induction of immunological tolerance (e.g., stem cell plus organ grafts from the same donor) will generate some period of chimerism with the potential for systemic infections or graft-vs-host disease.

The risk for infections in xenotransplantation is unknown without human studies—Clinical data will drive improvements in the production of source animals including genetic modifications and improve surveillance strategies for subsequent recipients. New microbiological assays will be developed to identify or exclude potential human pathogens from breeding herds and for the diagnosis of such organisms in humans. Next generation sequencing from samples from xenograft recipients may identify unsuspected microbes – their clinical significance is unknown in the absence of clinical data. However, significant progress has been made in understanding of approaches to and management of potential infections in xenotransplantation.

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Table 1:

Deployment of Microbiological Assays in Xenotransplantation

Assay Type	Screening Source Animals	Xenograft Recipients Monitoring	Xenograft Recipients – Symptomatic Infection or Increased Risk*	Healthy Contacts of Recipient
Cultures (Active Infection)	X		X	
Serology (Past Exposures)	X	X	+/−	X
Molecular Assay or Antigen Detection (Active Infection)		X	X	+/−
Agnostic, metagenomic Sequencing (Active Infection)		X	X	

*Increased risk may be associated with treatment of graft rejection or intercurrent viral infection.

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Table 2:

Exclusion List: Porcine Organisms to Consider (adapted from [4])

Viruses	
Porcine Endogenous Retrovirus (PERV) A, B, C, AC	Porcine lymphotropic herpesvirus (PLHV)
Porcine Adenovirus	Porcine Teschovirus
Encephalomyocarditis Virus	Rabies virus
Hepatitis E Virus	Swine influenza virus
Porcine Cytomegalovirus	West Nile Virus
Porcine Hemmagglutinating encephalomyelitis	
Bacteria:	
Mycobacteria spp.	Shigella
Pathogenic E. coli	Yersinia
Campylobacter	Leptospira spp.
Salmonella (cholerasuis, typhimurium)	Listeria spp.
Parasites:	
Toxoplasma gondii	Echinococcus spp.
Cryptosporidium parvum	Trichinella spiralis
Strongyloides	Microsporidium
Trypanosoma species	

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Table 3:

Recipient Monitoring for Viral Infection Post-Xenotransplantation (adapted from [4])

Virus Name – non-commercial testing	Testing Method
Porcine Endogenous Retrovirus (PERV) A, B, C, AC	Qualitative and Quantitative (QNAT) Nucleic Acid Testing (NAT); Antibody-based tests (serology, ELISA, Western Blot)
Porcine Lymphotropic Herpesvirus Type 2 (PLHV-2)	QNAT
Porcine Cytomegalovirus (PCMV)	NAT; Antibody-based tests
Human Cytomegalovirus (HCMV) – per protocol	QNAT
Human Epstein-Barr virus (EBV) – per protocol	QNAT