Infectious Risk and the Challenge of Safe Xenotransplantation

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- **Relationships with commercial interests:**
  - **Grants/Research Support:** NIH (PO1, T32, R01, U01)
  - **Consulting Fees:** Elion, Eledon, Jura, Well Medical, eGenesis, Markana, United Therapeutics, Bain, CLD Inc, OM1.
  - **Other:** Employee of Mass General Brigham Healthcare Inc (owner of MGH)
  - Ad hoc consultant on Xenotransplantation (SGE) for FDA Cellular, Tissue, and Gene Therapies Advisory Committee (CTGTAC), WHO, and Harvard Medical School.
- My presentation does not include discussion of off-label or investigational use drugs.

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The Growing Problem of Organ Shortages

Every ten minutes, someone is added to the USA transplant waiting list.

On average, ~20 people die in the USA each day while waiting for a transplant – many more worldwide.

In the United States:
105,766 *people are waiting for an organ transplant*
(Likely a big underestimate)
First 6 months of 2022:* 12,104 *donors* & 24,414 *transplants
But
~7000 DIED WAITING FOR AN ORGAN LAST YEAR

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Alternative Therapies for Organ Failure:

- Better disease prevention (hypertension, diabetes, smoking, autoimmune diseases …)
- Treatments (e.g., Hepatitis C)
- Gene therapies (CRISPR)
- Stem cell therapies (stem cell derived islets, organ repair therapies)
- Improved organ transplantation and survival
  - Better immunosuppression
  - Organ perfusion systems and perfusion solutions
- Artificial organs – re-engineered, decellularized organs or 3D printed scaffolds?
- ***Animal organs***? Immunologic and Metabolic Barriers

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Why Pigs?

ADVANTAGES OF SWINE

- Breeding characteristics well described for commercial use
- Can size to humans
- Experience with inbreeding and genetic manipulation → reagents for study of cell surface antigens
- Advanced genetics (CRISPR) → can derive new strains of swine with desired characteristics
- Sequence data are available from some important viruses and veterinary lab experience with microbiology, vaccines
- Resistance to HIV, HBV, HCV

DISADVANTAGES

- Preformed natural antibodies to α–Gal sugars (hyperacute rejection)
- Metabolic incompatibilities
- Histo-incompatability for humans

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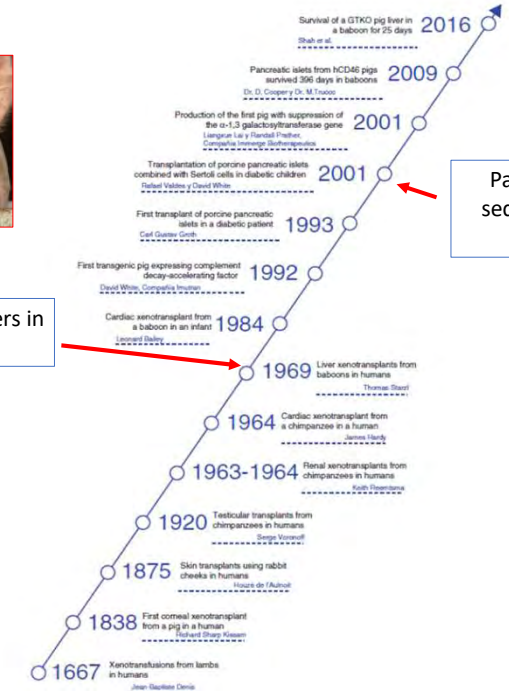
Starzl Team implants baboon livers in humans.

2021: US surgeons successfully test pig kidney transplant in deceased recipient



Patent awarded for cloning and sequence of Porcine Endogenous Retrovirus (JAF)

2022: US surgeons implant genetically modified pig heart in human who lived for 2 months

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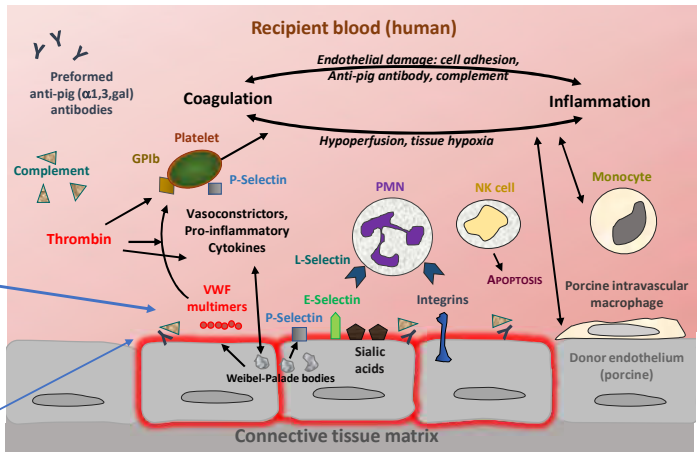
Challenges: Mechanisms of Porcine Endothelial Injury by Human Blood

Preformed human anti-pig antibodies bind to porcine endothelium, triggering complement binding and Fc-receptor–mediated ligation of platelets and leukocytes and upregulation of adhesion molecules on both adherent formed blood elements and inflamed endothelium.

Complement cascade activation, adhesion of human platelets to porcine endothelium → prothrombotic, proinflammatory milieu → loss of vascular barrier function and organ failure.

Dysregulated Anticoagulation by Porcine Endothelium with Human Blood



Lack of non-self signals → NK, T-cell activation against graft, may lack immune function to protect vs. infection.

GP = glycoprotein; ICAM, intercellular adhesion molecule; IL-6, interleukin-6; PMN, polymorphonuclear; PSGL-1, P-selectin glycoprotein ligand 1; TNF, tumor necrosis factor; and vWF, von Willebrand factor

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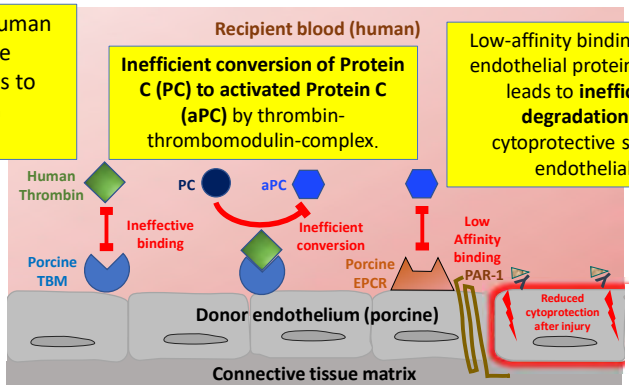
Dysregulated Anticoagulation for Porcine Endothelium with Human Blood

Ineffective binding of human thrombin by porcine thrombomodulin leads to **reduced thrombin deactivation.**

Inefficient conversion of Protein C (PC) to activated Protein C (aPC) by thrombin-thrombomodulin-complex.

Low-affinity binding of aPC to porcine endothelial protein C receptor (EPCR) leads to **inefficient thrombin degradation** and reduced cytoprotective signaling through endothelial cell PAR-1.



Porcine endothelium exposed to human blood activated by binding of anti-pig antibodies → prothrombotic environment.

Amplification of blood clotting by multiple factors: *ineffective neutralization of human thrombin* by porcine thrombomodulin, *poor conversion of protein C (PC) to activated PC (aPC)* by thrombin-thrombomodulin complex, and low-affinity binding of human aPC to porcine endothelial protein C receptor (EPCR), which in turn leads to ***inefficient thrombin degradation*** and reduced cytoprotective signaling via PAR-1 (endothelial cell proteinase-activated receptor).
Genetic modifications include: expression of human thromboregulatory proteins including human thrombomodulin and human endothelial protein C receptor (EPCR) & human tissue factor pathway inhibitor.

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Mechanistic Barriers to Pig-to-Human Xenotransplatation

Phenomenon	Kinetics	Mechanisms
Hyperacute rejection	Minutes to hours	Preformed antibody, complement, clot formation, endothelial injury
Initial xenograft dysfunction	Minutes to hours	Metabolic/physiologic? Immune? Ischemia/reperfusion?
Delayed Xenograft rejection	Days – Weeks	Preformed antibody rebound, elicted antibody
Chronic Rejection	Weeks	Elicited immunity, dysregulated coagulation

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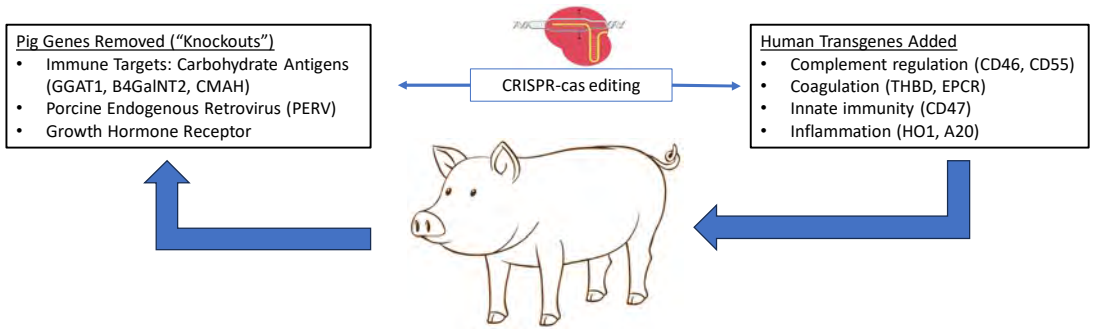
Figure 1. Advances in genetic engineering have allowed creation of pigs with advantages in terms of infection, (virus deleted), immunology (less rejection), coagulation (less blood clotting), size, and inflammation. Breeding of source animals in biosecure facilities allows screening for potential pathogens.



Screening/Exclusion Criteria ("*Designated Pathogen Free*")
- Pig-specific viruses (e.g., porcine cytomegalovirus, porcine circovirus, porcine endogenous retrovirus, porcine lymphotropic herpesvirus)
- Pathogens of pigs and humans (e.g., *Toxoplasma gondii*, influenza, Hepatitis E, Nipah, *Strongyloides stercoralis*)
- Similar to pathogens of immunocompromised hosts (e.g., porcine adenovirus)
- Unknown organisms (consider metagenomics)

Fishman, 2024

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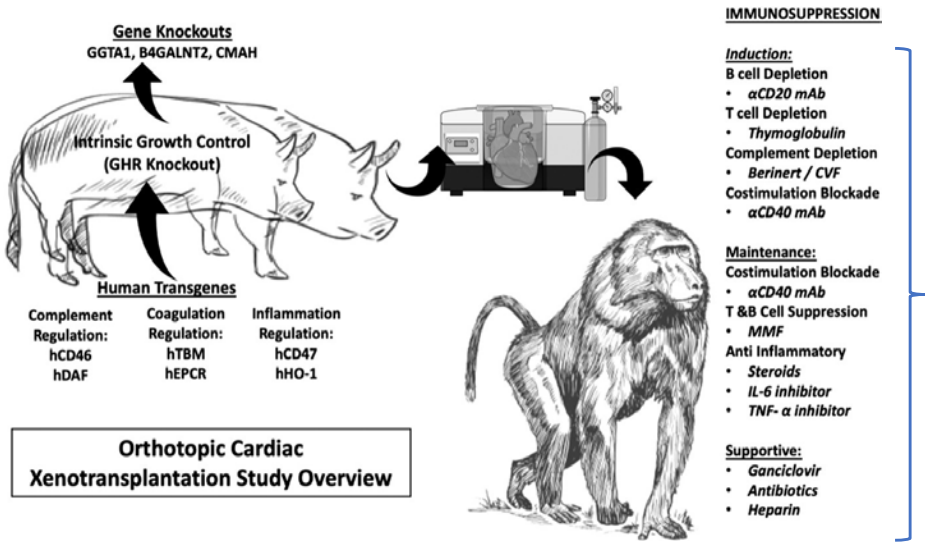

Orthotopic Cardiac Xenotransplantation Study Overview

Xenotransplantation, Volume: 29, Issue: 3, First published: 31 March 2022, DOI: (10.1111/xen.12744)

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Optimizing safety in clinical xenotransplantation: Infectious Disease Risks

The risk for infection depends on the *nature, intensity, and duration of immunosuppression* as well as on infectious exposures (epidemiology).

Each clinical trial will be a "**package**" of:

- Specific organs required by recipient
- Patients with various latent infections and exposures
- Source swine with different genetics and breeding characteristics (microbiological screening)
- Various immunosuppressive regimens
- Various approaches to monitoring recipient

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Infectious Disease Gaps to enhance safety and efficacy of clinical xenotransplantation (*requires human data*)

- **Potential zoonoses**: **Develop microbiologic surveillance and *exclusion criteria* for pig production and to monitor recipients and contacts.**
  - **Which organisms** infect human cells or cause clinical syndromes in immunosuppressed recipients?
  - **Microbiolo** PERV infect non-transforme
  - **Approach**
  - Any impact
- **Diagnostic as**
  - **Which ne**
  - **Monitoring** cts?
  - Databank (
- **Management of novel immunosuppressive regimens**? And risk for infection?
- **Infection Control**: How and who?
  - Protocols for managing "sick" recipients. (Isolation?)
  - Protocols for staff and others in contact with pigs and recipients (sample archiving)
  - Handling of surgical equipment and sterilization of rooms
- **Unknowns** (few data on infectious risks) → Ethical issues for informed consent

Its not complicated!

Can organisms from pigs cause significant infection in humans?

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Bulk Of Confirmed Human Cases Of Swine Flu In US Since 2011 Have Been Connected To Agricultural Showcases (New York Times July 25, 2023)

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Take Home Message:
Considerations for Xenotransplantation in Clinical Trials

- ✓The goal is to **define parameters for safety in clinical xenotransplantation** (focus on pig model) – e.g., *As safe as allotransplantation relative to infectious risk?*
- ✓**Develop assays** and preventative strategies for potential human pathogens (focus on viruses, pig-specific pathogens)
- ✓Characterize the **biology of potential pathogens in clinically relevant models** (immunocompromised hosts *in vivo*)
- ✓**Science**: Understand the impact of immune suppression, tolerance induction, and genetically engineered source animals on infectious risk

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Framework: Categories of Potential Human Pathogens Resulting from Xenotransplantation

- **Common Human Pathogens of Allotransplant Recipients** (EBV, CMV, herpes simplex virus, varicella zoster virus, Apergillus, *Listeria*, Mycobacteria)
  - **Specific serologic tests and microbiological assays are generally available**
  - **Therapies generally available**
- **Traditional Zoonoses:** well-characterized *clinical syndromes of humans* (*Toxoplasma)*
  - **Specific microbiological assays are generally available**
  - **Therapies generally available**
- **Species-specific agents:** organisms thought to be incapable of causing infection outside the xenograft (e.g., porcine CMV, Porcine lymphotropic herpesvirus, circovirus)
  - **Some specific microbiological assays are available; few validated assays available for use in humans**
  - **Impact of infection limited to xenograft and unknown host response to infection**
- **Potential pathogens:** Organisms of broad "host range" which may spread beyond the xenograft (adenovirus, influenza, coronaviruses, actinomyces, mycobacteria)
  - **Specific microbiological assays are available for use in humans; may not be standardized for porcine strains; limited therapies available**
- **New/Unknown pathogens:** Organisms not known to be human pathogens
  - **Unknown pathogenicity within the new host (e.g., retroviruses)**
  - **Unknown clinical syndromes; microbiologic assays limited; some therapies**

See: Fishman, JA. *Xenotransplant, 1994, 47-57; Kidney International, 1997, 51(supp): 41-45; Xenotransplant 2007, 14:349-352; J Cardiac Surg, 2001, 16: 363-373.*

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Viruses of Swine: *Potential Causes of Infection or Adverse Effects in Human Xenograft Recipients?*

- *Adenovirus sp.*
- African swine fever
- Encephalomyocarditis virus
- ***Influenza virus (swine, avian, human)***
- Lymphocytic choriomeningitis virus (LCMV)
- *Nipah (Hendra-like) respiratory virus of humans*
- *Menangle virus (fruit bat and swine, human infection mild, + serology)*
- ***Porcine circovirus 1, 2 and 3 – nonproductive infection in vitro, in vivo (Graft infection)***
- Porcine coronavirus
- ***Porcine cytomegalovirus (PCMV)***
- ***Porcine endogenous retrovirus (PERV)***
- ***(Porcine) Hepatitis E virus – HEV genotypes 1 and 2 common in humans***
- Porcine lymphotropic herpesvirus (PLHV-1, -2, -3)
- Porcine parvovirus (PPV) – ?
- Porcine polyomavirus
- Porcine Reproductive and Respiratory Syndrome virus (increased by coinfection with *Streptococcus suis*) - ?
- Pseudorabies virus
- *Rabies virus*
- Rotavirus
- **Anellovirus /Torque tenovirus -?**

Need consistent surveillance plan in source animals and recipients

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"Designated-Pathogen-Free" Miniature Swine: Potential Human Pathogens?

Bacteria:	Parasites:	Viruses:
Brucella suis	Ascaris suum	*Adenovirus* (porcine)
Leptospira spp.	Cryptosporidium parvum	*Circovirus 1, 2 and 3 (vaccine)*
Listeria monocytogenes	Isospora sp.	Porcine Cytomegalovirus
Mycobacterium bovis	Neospora	Encephalomyocarditis virus
Mycobacterium tuberculosis	Strongyloides ransomi	*Hepatitis E Virus*
Mycobacterium avium - intracellulare complex	Toxoplasma gondii	*Influenza virus* (porcine and human)
Mycoplasma hyopneumoniae (lungs?)	Trichinella spiralis	Porcine Lymphotropic Herpes (PLHV)?
Salmonella typhi, typhimurium, cholerasuis		Porcine Reproductive and Respiratory Syndrome Virus
Shigella		Nipah (Hendra-like) and Menangle virus
Trypanosoma spp.		Porcine Parvovirus
	Fungi:	*Porcine endogenous retrovirus (A,B,C, AC)*
	Aspergillus species (colonized or lesions)	Porcine Hemmagglutinating encephalomyelitis
	Candida species (Lesions)	Porcine Teschovirus
	Cryptococcus neoformans	Pseudorabies / Rabies
	Histoplasma capsulatum	*Rotavirus*

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Need validated assays for studies of swine organisms in human recipients of xenografts

Serology – useful for screening (past exposures)

Cultures – routine and viral

Single or Multiplex Molecular (Quantitative PCR) Assays for potential human pathogens:

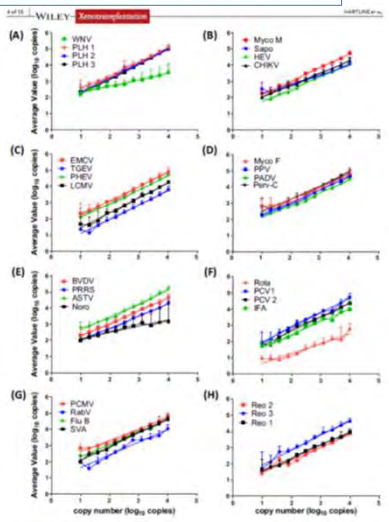
- Screening – generally <u>not</u> useful for this indication
- Monitoring – for activation
- Diagnosis of acute infectious syndrome

Metagenomics – whole genome sequencing

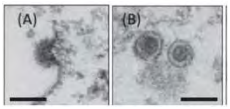
- Currently available databases are for human pathogens
- Requires larger database of pig pathogens
- Reverse transcribed for RNA viruses
- Pig genome data useful for correction for circulating pig cells and cell-free DNA/RNA

Others:

- **ELISA**, Western Blot, Serologies (vs. viral proteins)
- **Electron Microscopy**



Hartline CB et al. Xenotrans.2018; 25:e12427



EM From Denner, J. Xenotransplantation. 2020;00:e12594. https://doi.org/10.1111/xen.12594

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How does one get into this field?

" I have some sick pigs."

David Sachs, MGH, circa 1993.

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Xenotransplantation and Xenosis: Are the Risks Enhanced?

- **Transplant Bypasses Host Defenses**: no "vector" needed for transmission
- Increased **intensity of immunosuppression required?**
- **Xenograft provides ecologic niche** in the body (culture plate) for swine-specific organisms.
- Potentially **protected site** from cellular immunity due to MHC-mismatch
- **Organisms** not detected by current clinical microbiologic assays, not identified as human pathogens, no pre-existing immunity, or "**xenotropic**" (causing disease in non-native host)
- **Swine organisms: New clinical syndromes**? Non-recognition of infection.
- **Genetic modification** of donor or treatment of recipient may alter susceptibility to and manifestations of infection

Fishman, JA. Infection and Xenotransplantation: Assessing the Risks. Clin. Micro. News 1998, 20:141-143; **Fishman, JA**. Infection and Xenotransplantation: Developing strategies towards clinical trials. Graft, 1998, 1(5): 181-185.

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Source Animal Health (veterinary practice)

- Organisms to be excluded based on regulations or animal health
- Goal is to minimize use of antimicrobial agents in herd (selection of resistant organisms)
- Standard vaccinations
- Environment (breeding and transportation) and feed, human and animal (and insect) contacts regulated to exclude introduction of infection
- *Novel risks (e.g., susceptibility) due to genetic manipulation?*

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Viral Activation in Transplantation

Common factors in the activation of latent herpesviruses and retroviruses are present in both allo- and xeno-transplantation

- **Immune Responses** (graft rejection)
- **Immunosuppression** (T-cell depleting antibodies)
- **Infection/Inflammation/Cytokines**
- **Cytotoxic Agents**
- **Radiation Therapy**

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Xenopathogen Surveillance

Table S3: Results of Commercial Pathogen Screening of Source Pig

Test	Sample	Assay	Result	Date
Hepatitis E	Feces	Real-time PCR	Negative	1/7/22
Herpes virus gamma	Buffy coat	PCR	Negative	1/7/22
Influenza A	Nasal swab	Real-time PCR	Negative	1/7/22
Mycoplasma hyopneumoniae	Nasal swab	Real-time PCR	Negative	1/7/22
Porcine cytomegalovirus	Nasal swab	Real-time PCR	Negative	1/7/22
Porcine circovirus type 2	Serum	Real-time PCR	Negative	1/7/22
Porcine circovirus 3	Serum	Real-time PCR	Suspect [Ct 39]	1/7/22
Porcine Epidemic Diarrhea virus (S gene)	Feces	Real-time PCR	Negative	1/7/22
Porcine deltacoronavirus	Feces	Real-time PCR	Negative	1/7/22
Transmissible Gastroenteritis virus	Feces	Real-time PCR	Negative	1/7/22
Porcine reproductive and respiratory syndrome virus (PRRSV)	Serum	Real-time PCR	Negative	1/7/22
Porcine endogenous retrovirus A	Buffy coat	PCR	Positive [Ct 20]	12/21/21
Porcine endogenous retrovirus B	Buffy coat	PCR	Positive [Ct 22]	12/21/21
Porcine endogenous retrovirus C	Buffy coat	PCR	Negative	12/21/21

Patient developed PCMV despite negative PCR!

Griffith, BP e al. 2022. NEJM; 387:35-44.

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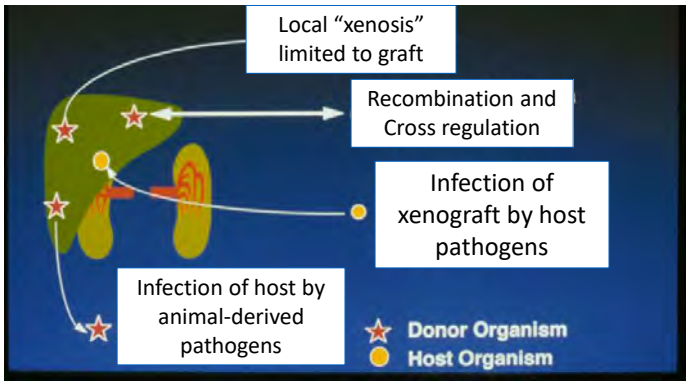
Sites of infection in xenotransplantation: Model for Donor-derived Infection



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Known Effects of Porcine Viruses in Immunosuppressed Baboons

- **Porcine cytomegalovirus (PCMV)** (also called Porcine roseolovirus, Suid betaherpesvirus 2) – species specific (no human infection)
  - **Infection of graft with endothelial activation (tissue factor) → systemic neutropenia, coagulopathy, graft rejection**
  - Can be bred out of colony (easily reintroduced)
  - Poorly susceptible in vitro to common antivirals
- **Porcine lymphotropic herpesvirus (PLHV)** – 3 known viruses → lymphoma-PTLD in swine
  - *No evidence of disease in baboons (species-specific)*
- **Porcine Circovirus** (1,2,3) – pneumonitis, lymphadenitis in swine
  - No disease in baboon, human, possible infection of xenograft?
- **Coronaviruses: Pigs not infectable by SARS-CoV-2** & do not carry CoV's pathogenic for humans. However, pigs carry angiotensin-converting enzyme 2 (ACE2) the common cellular receptor for spike (S-glycoprotein) of SARS-CoV-2. (Schlottau K, et al. Lancet Microbe 2020;1(5):e218-e225. DOI: 10.1016/S2666-5247(20)30089-6.)
- **PERV – Porcine Endogenous Retrovirus (A, B, C, AC)**
  - No effects identified in swine; cell surface receptors exist in humans
  - No productive infection in baboons (lack functional receptor)
  - Unknown virulence in human host (*carry functional receptors; replication only on transformed cells*)
  - Susceptible in vitro to available antivirals

All pig-to-primate studies performed in collaboration with lab of David Sachs. All studies generously supported by NIH-NIAID.

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Likely Pig-specific organisms

- **Porcine Circovirus (PCV)** – *worldwide distribution* of *three species*: PCV 1 (nonpathogen), PCV 2 (post-weaning multi-systemic wasting syndrome, PMWS), and PCV 3 (important swine pathogen). Non-enveloped spherical particles with a single-stranded circular small DNA genome.
  - PCV3 may be limited pathogen (colonizer?) without other coinfecting viruses such as PCV 2 or porcine reproductive and respiratory syndrome virus (PRRSV). Associated with porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, cardiac and multisystemic inflammation. Not reported in immunosuppressed swine.
  - PCV 3 in xenotransplantation reported in Göttingen Minipigs (GöMP) knocked out for α1,3-galactosyltransferase (GT-KO), and expressed human membrane cofactor protein (CD46) and human thrombomodulin (hTM)
  - Maintenance immunosuppression based on mycophenolate mofetil, CD40/CD40L costimulation blockade (monkey-specific anti-CD40 monoclonal antibody or PASylated αCD40L Fab), and corticosteroids in addition to an induction therapy with an anti-CD20 antibody and anti-thymocyte-globulin
  - Higher viral loads were found in animals with longer survival times, ***indicating the replication of the virus*** per the authors but no demonstration of infection made.
- **No infection of human cells in vitro** (PCV 3-positive pig PBMCs stimulated with a T cell mitogen cocultured with human 293 cells for various time points, no transmission was observed)

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PCV3 in Cardiac xenografts: Rising Viral Load with Pig-Specific Organism

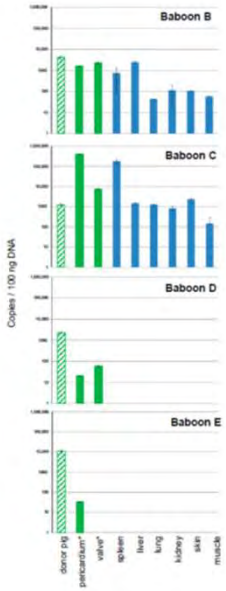

Figure 1. Detection of PCV3 in the organs of four PCV3-positive donor pigs (green hatched), in the transplanted pig heart after its removal at the end of the study (green) and in different organs of the baboon recipient (blue).

Comment: Baboons with longer survival had greater viral loads. No demonstration of infection of baboon cells.

Krüger L et al. Transmission of Porcine Circovirus 3 (PCV3) by Xenotransplantation of Pig Hearts into Baboons. Viruses. 2019 Jul 16;11(7):650. doi: 10.3390/v11070650. PMID: 31315245; PMCID: PMC6669873.

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Porcine lymphotropic herpesvirus (PLHV)

- Porcine lymphotropic herpesviruses (PLHV 1, PLHV 2, PLHV 3) are gamma herpesviruses common in swine.
- PLHV has some characteristics of both Epstein-Barr virus (EBV, cause of PTLD) and Kaposi's sarcoma virus (KSHV, sarcoma).
- PLHV 1 causes post-transplantation lymphoproliferative disease (PTLD) with immunosuppression and experimental transplantations in mini-swine.
- PLHV 1 not transmitted to baboon recipients in preclinical studies and not activated in graft. Not excluded by early weaning.

- Mueller NJ, Livingston C, Knosalla C, Barth RN, Yamamoto S, Gollackner B, Dor FJMF, Buhler L, Sachs DH, Yamada K, Cooper DKC, **Fishman JA**. Activation of Porcine Cytomegalovirus but not Porcine Lymphotropic Herpesvirus in Pig-To-Baboon Xenotransplantation, J Infect Dis, 2004, 189:1628-1633.
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Porcine Cytomegalovirus (PCMV)

- **Replicates only in pig cells/tissues** – can see simultaneous PCMV in pig cells and BCMV in baboons
- **Provokes consumptive coagulopathy, activation of tissue factor, graft rejection in xeno-baboons** –prevented by removal of PCMV.
- Diagnostic tools developed (Serology, QPCR and culture)
- Relatively resistant to ganciclovir therapy (compared with human or baboon CMV)
- Some in vitro data suggest that human CMV has limited capacity to infect porcine vascular endothelial cells in vitro (Mueller et al)
- Can be removed from herd by early weaning or Caesarian section but easily reintroduced

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- ***Fishman JA**, Sachs DH, Yamada K, Wilkinson RA*. Absence of Interaction between Porcine Endogenous Retrovirus and Porcine Cytomegalovirus in pig-to-baboon renal xenotransplantation in vivo. Xenotransplant 2018. 2018 Sep;25(5):e12395. doi: 10.1111/xen.12395. Epub 2018 Apr 6. PMID:29624743.

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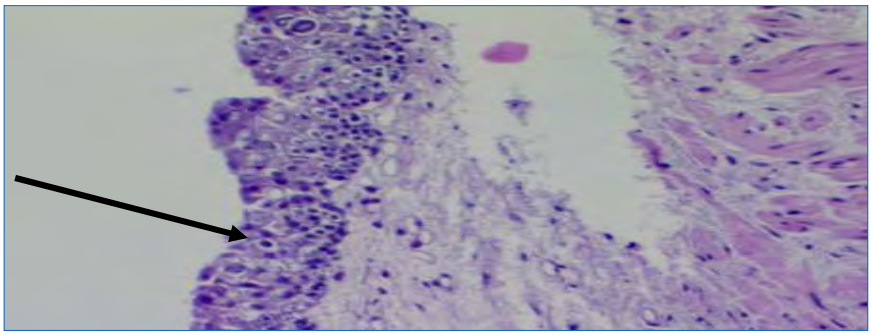
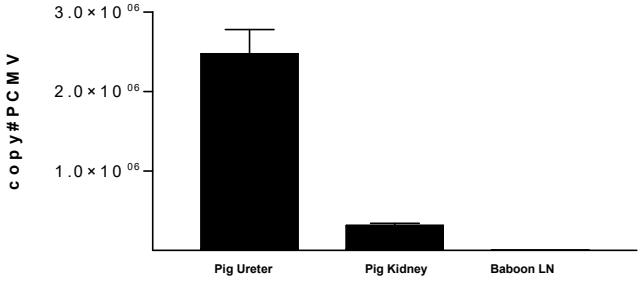
Host-specific PCMV Infection





Mueller et al. PCMV in pig-to-primate xenotransplantation

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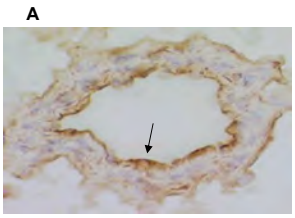
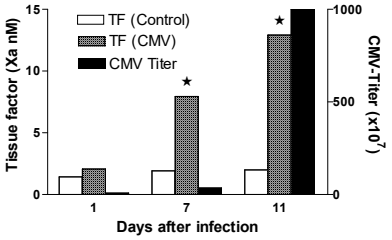


Bernd Gollackner et al.

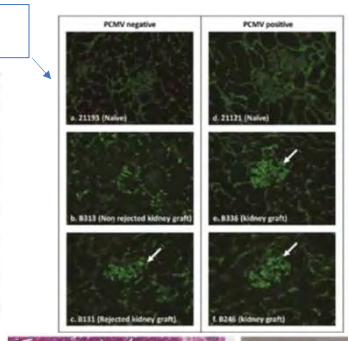


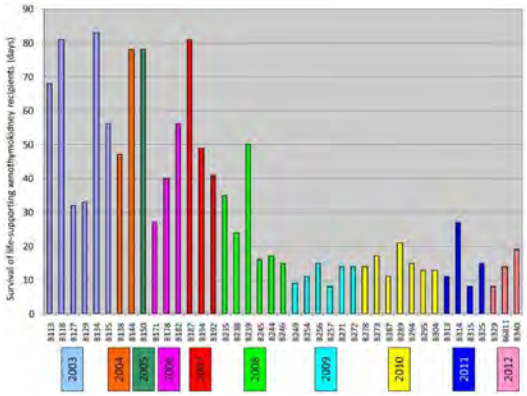
*PCMV induces endothelial cell activation in vitro and in vivo with procoagulant expressio*n: porcine tissue factor (pTF) upregulation in CMV-infected PAEC. White bars show TF in control cells, shaded bars show upregulation in infected cells. Immunohistochemistry for porcine TF of a pig kidney excised on day 29 for consumptive coagulopathy.

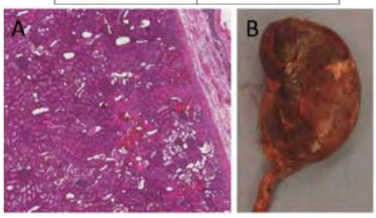
32

PCMV & Xenograft Rejection

Class II Expression







PCMV+ xenograft with acute rejection

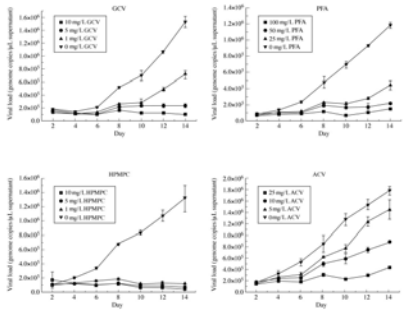
Yamada K et al. *Transplantation*. 2014;98(4):411–418.

33

Susceptibility of PCMV (clinical isolate): Reduction (%) of PCMV qPCR

Concentration of drug (microM)	300	100	30	10	3	1
Acyclovir uM	toxic	95	71	52	n/c	
Ganciclovir uM	toxic	toxic	toxic	92	49	
Cidofovir uM	toxic	toxic	toxic	98	97	93
Foscarnet uM	95	90	n/c	n/c	n/c	n/c
Leflunomide (A77 1726) ug/ml	toxic	toxic	n/c	n/c	n/c	n/c

n/c, no change



Susceptibility of PCMV to antiviral drugs: cell-free virus in supernatant over a 14 day period (Triplicates +/- SEM).

Jacqueline F. L. Fryer et al. J. Antimicrob. Chemother. 2004;53:975-980
GCV, ganciclovir; PFA, foscarnet; HPMPC, cidofovir; ACV, acyclovir.

34

Porcine Endogenous Retrovirus (PERV): A Long Story

- Todaro (1974): C-type retrovirus from PK-15
- Suzuka (1985): C-type retrovirus from swine malignant lymphoma (Tsukuba-1 virus)
- Kaeffer (1990): Tsukuba-1 causes lymphomas in wild boar (but not infective for human cell lines or mice; has RT activity in vitro)
- Le Tissier (1997): Two classes of PERV from PK-15 cell line (PERV A and B) and Patience (1997): PK-15 virus infective for transformed human cell lines
- Akiyoshi & Fishman (1997): **Full-length sequence of Tsukuba-1 and PERV from *normal pig cells* (PERV C)** - constitutive expression; copy number & distribution vary by pig strain. (Akiyoshi DE, Denaro M, Zhu H, Greenstein JL, Banerjee P, **Fishman J**. Identification of a Full-length cDNA for an Endogenous Retrovirus of Miniature Swine. J. Virology, 1998, 72:4503-4507.)

35

Infectious risks of xenotransplantation cannot be assessed in the absence of clinical trials.

What do we need to learn?

36

THE BOSTON GLOBE ★ FRIDAY, JANUARY 17, 1997 A7

Britain to propose ban on animal-to-human transplants

LONDON – The government, worried about strange viruses and other health issues, said yesterday it would ban animal-to-human organ transplants until scientists know more about their safety.

Laws will be enacted if necessary to prevent any human trials of such transplants, known as xenotrans-

plants, a spokesman said.

"It is essential that the risks associated with xenotransplantation are better understood before the technique is used on human patients," Health Secretary Stephen Dorrell said in a statement.

He said laws governing xenotransplants would be proposed as soon as possible.

"It is the right decision. There

Risk of spread of viruses is cited

are risks, risks which go beyond the animal patient," Robin Weiss of the Institute of Cancer Research told BBC television.

Specialists say viruses not harmful to the host animal would be difficult to screen for and could kill people. Once infected, a transplant patient could infect many others.

For instance, the HIV virus that causes AIDS is believed to have originated in monkeys or apes.

Last year the Nuffield Council on Bioethics, an advisory group made up of doctors, scientists and other specialists, said xenotransplants would be ethically acceptable if precautions were taken to make sure

humans did not risk infection and animals were not abused.

David Shapiro, executive secretary of the council, said Britain was probably the first country to make such a move.

"Both in the US and in the UK we are gearing ourselves up for it as we are likely to be the first two countries affected," he said. "Britain has taken a decision. The United States

is still in the process."

The US Institute of Medicine issued guidelines on possible animal transplants last fall but has not called for national regulation.

Animal welfare groups have urged that all animal-to-human transplants be banned. One group, Compassion in World Farming, said it feared that animals would be mistreated.

Dateline: 1997

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When will we have clinical xenotransplantation?

"When Jay Fishman stops writing papers!"

Thomas Starzl, M.D.

International Transplant Infectious Disease Congress, Orlando, FLA, 1997

38

Human-tropic, replication competent (HTRC) porcine endogenous retrovirus (PERV AC) *in vitro*

- Three types of PERV (A, B, C) exist that differ in *env* region and receptor binding. **PERV-A and PERV-B are capable of infecting human cells** and are present in the genome of most pigs. **PERV-C infects only pig cells** and is present in the genome of many, but not all pigs.
- PERV receptors are present on human cells. However, *productive infection of normal human cells by PERV by normal porcine tissues has not been demonstrated*
- *In vitro* infection of **permissive human cell line** (HEK297, adenovirus transformed) by PERV A and B requires high titer virus or direct contact with pig cells.
- Replication efficiency increases with **passage in vitro** → produces a recombinant PERV A and C (**HTRC PERV AC**) with improved replication. PERV AC is present in genome of some normal swine
- **No human infection demonstrated in recipients of alginate encapsulated porcine islets or >200 individuals exposed to pig cells/tissues.**

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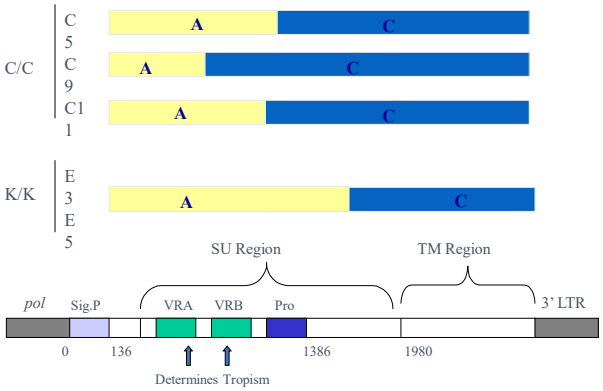
HIGHLY REPLICATION COMPETENT PERV ARE RECOMBINANT VIRUSES *IN-VITRO*



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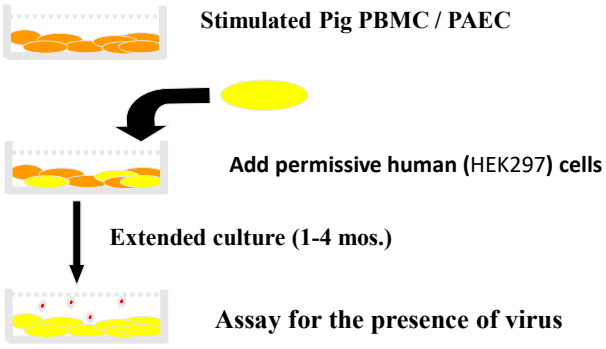
PERV TRANSMISSION ASSAYS



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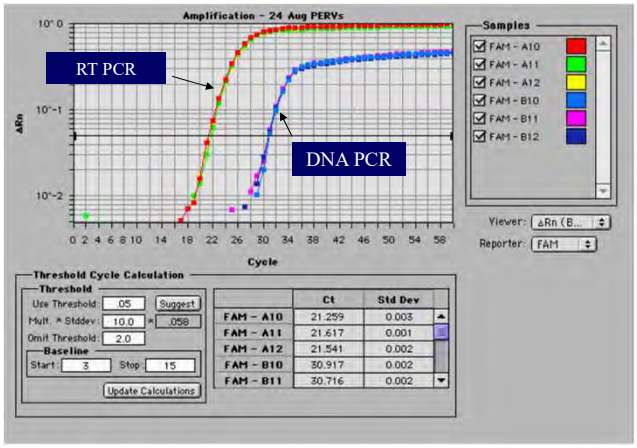
PERV pol Taqman PCR



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Porcine Endogenous Retroviruses (PERV)

Enveloped RNA viruses can integrate into the genome of infected individuals as proviruses where they can remain as fragments or full length copies, potentially reactivated.

Many endogenous type C retroviruses are present in all reptiles, birds and mammals without effect on host species – but with capacity to infect across species (xenotropic host range)

Three replication-competent subtypes of PERV: PERV-A, PERV B, and PERV C. PERV A and B are polytropic, capable of infecting both porcine and human cells (receptors present in humans, not in baboons)

PERV A/C recombinants represent the PERV A sequence for receptor-binding (SU region in the env) and remaining sequence coming from PERV C.

Infectivity is demonstrated ONLY on adenovirus transformed human cells (HEK 293). No infection (virus or serology) has been demonstrated in individuals with exposure to pig tissues. Erroneous publication suggested infection of humanized mice after islet transplantation.

PERV AC develops in vivo in swine as well as in vitro. PERV A/C is significantly more infective for HEK-293 cells than PERV A.

Wilhelm M, **Fishman JA**, Pontikis R, Aubertin A-M, Grierson DS, Wilhelm FX. Susceptibility of Recombinant Porcine Endogenous Retrovirus Reverse Transcriptase to Nucleoside and Non-nucleoside Inhibitors. Cellular and Molecular Life Science, 2002, 59:2184-90.

Wood JC, Quinn G, Suling KM, Oldmixon BA, Van Tine BA, Cina R, Arn S, Huang CA, Scobie L, Onions DE, Sachs DH, Schuurman H-J, **Fishman J**, Patience C. Identification of exogenous recombinant human-tropic porcine endogenous retrovirus. J. Virol 2004; 78:2494-2501.

Yang YG. Wood JC. Lan P. Wilkinson RA. Sykes M. **Fishman JA**. Patience C. Mouse retrovirus mediates porcine endogenous retrovirus transmission into human cells in long-term human-porcine chimeric mice. Journal of Clinical Investigation. 114(5):695-700, 2004

*Martin SI, Wilkinson RA, **Fishman JA**.* Genomic presence of recombinant porcine endogenous retrovirus in transmitting miniature swine. Virology J., *Virology Journal* 2006, 3:1743-422(http://www.virologyj.com/content/3/1/91)

Nicolas C. Issa NC, Wilkinson RA, Griesemer A,Cooper DKC, Yamada K, Sachs DH, **Fishman JA**. Absence of Replication of Porcine Endogenous Retrovirus and Porcine Lymphotropic Herpes Virus type 1 with Prolonged Pig-Cell Microchimerism after Pig-to-Baboon Xenotransplantation. J. Virol, 2008, 82(24):12441-8.

Yang L, Güell M, Niu D, George H, Lesha E, Grishin D, Aach J, Shrock E, Xu W, Poci J, Cortazio R, Wilkinson RA, **Fishman JA**, Church G. Genome-wide inactivation of porcine endogenous retroviruses (PERVs). Science DOI: 10.1126/science.aad1191 Online October 11 2015

Argaw, T., Colon-Moran, W., Wilson, C., Susceptibility of porcine endogenous retrovirus to antiretroviral inhibitors. Xenotransplantation, 2016. 23(2): p. 151-158

43

Human-tropic, replication competent (HTRC) porcine endogenous retrovirus (PERV AC) *in vitro*

- Three types of PERV (A, B, C) exist that differ in *env* region and receptor binding. PERV-A and PERV-B are capable of infecting human cells and are present in the genome of most pigs. PERV-C infects only pig cells and is present in the genome of many, but not all pigs.
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- Replication efficiency increases with **passage in vitro** → produces a recombinant PERV A and C (HTRC PERV AC) with improved replication.
- PERV AC is also present in genome of some normal swine
- No human infection demonstrated in recipients of alginate encapsulated porcine islets or >200 individuals exposed to pig cells/tissues.

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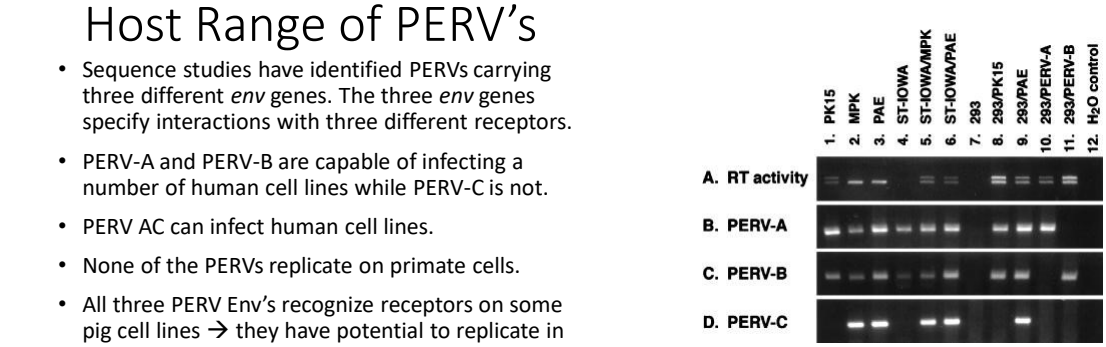
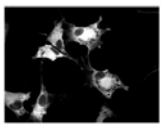
Host Range of PERV's

- Sequence studies have identified PERVs carrying three different *env* genes. The three *env* genes specify interactions with three different receptors.
- PERV-A and PERV-B are capable of infecting a number of human cell lines while PERV-C is not.
- PERV AC can infect human cell lines.
- None of the PERVs replicate on primate cells.
- All three PERV Env's recognize receptors on some pig cell lines → they have potential to replicate in pigs as well as likely in transplanted pig tissues.
- It is not known whether integrations occurring in pig tissues may occur at chromosomal sites favoring provirus expression or recombination.



Transmission of RT activity and PERV RNA expression. Cell supernatant was assayed for RT activity by a PCR-based method (gel A). RNA expression for PERV-A, PERV-B, and PERV-C was examined by RT-PCR (gels B, C, and D). ST-IOWA/MPK, ST-IOWA cells after cocultivation with MPK cells; ST-IOWA/PAE, ST-IOWA cells after cocultivation with PAE cells; 293/PK15, 293 cells infected with PK15 viral supernatant; 293/PAE, 293 cells after cocultivation with PAE cells.

B

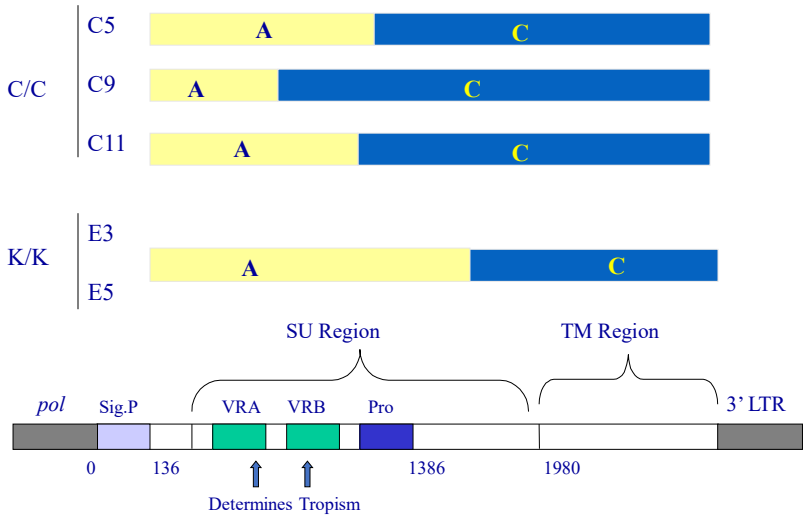


PERV receptor activity in vitro HuPAR-2EGFP protein is expressed at the plasma membrane of transduced SIRC cells. Intracellular protein, particularly in the perinuclear endoplasmic reticulum region, is also evident.

Takeuchi Y, Patience C, Magre S, et al. Host range and interference studies of three classes of pig endogenous retrovirus. *J Virol*. 1998;72(12):9986-9991. doi:10.1128/JVI.72.12.9986-9991.1998; Ericsson TA et al. Identification of receptors for pig endogenous retrovirus. Proc Natl Acad Sci U S A. 2003 May 27;100(11):6759-64. doi: 10.1073/pnas.1138025100. Epub 2003 May 9. PMID: 12740431; PMCID: PMC164520.

45

Human-tropic, replication competent (HTRC) porcine endogenous retrovirus (PERV AC) are recombinant viruses *in vitro*



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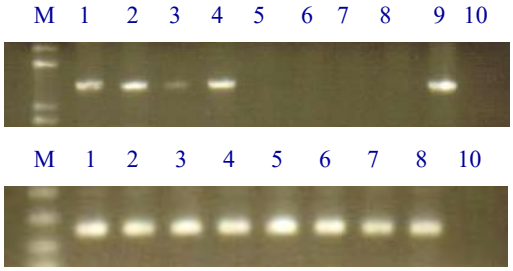
Recombinant PERV AC Exist in the Genome of Normal Mini-swine tissues: On-going infection

PCR with PERV-A SU region forward primers and PERV-C TM region reverse primers in the total cellular DNA harvested from tissues of swine (mitochondrial markers control)

The envelope glycoproteins of the mammalian type C retroviruses consist of two subunits, a surface (SU) protein and a transmembrane (TM) protein. SU binds to the viral receptor and is thought to trigger conformational changes in the associated TM protein that ultimately lead to the fusion of viral and host cell membranes.



1-4: transmitting swine

5-8: Gal-T-KO swine

9: Positive control

10: Negative control

Martin and Fishman Virology J., *Virology J* 2006, 3:1743-422

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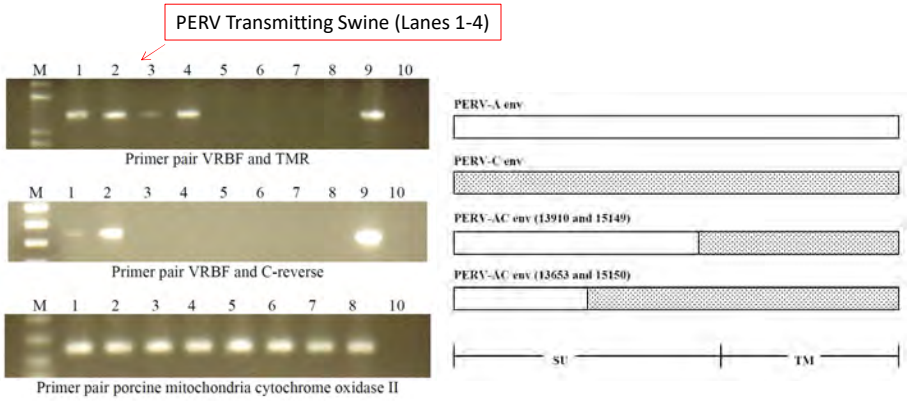
PERV AC Auto-infectious Cycle: Two different recombinant PERV-AC sequences were identified and sequenced from tissues (cellular DNA) of PERV-transmitting miniature swine and from cell cultures. **This was the first evidence of PERV-AC recombinant virus in porcine genomic DNA** that may have resulted from autoinfection following exogenous viral recombination.



Recombinant PERV AC Exist in the Genome of Normal Mini-swine tissues: On-going infection (from Martin and Fishman Virology J., *Virology J* 2006, 3:1743-422)

48

Quantification challenges

- Microchimerism – what does a high level of virus mean?

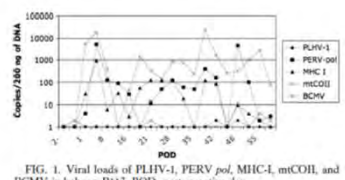


FIG. 1. Viral loads of PLHV-1, PERV *pol*, MHC-I, mtCOII, and BCMV in baboon B113. POD, postoperative day.

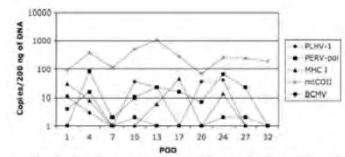


FIG. 2. Viral loads of PLHV-1, PERV *pol*, MHC-I, mtCOII, and BCMV in baboon B114. POD, postoperative day.

JOURNAL OF VIROLOGY, Dec. 2008, p. 12441–12448 Vol. 82, No. 24
0022-538X/08/$08.00+0 doi:10.1128/JVI.01278-08

Absence of Replication of Porcine Endogenous Retrovirus and Porcine Lymphotropic Herpesvirus Type 1 with Prolonged Pig Cell Microchimerism after Pig-to-Baboon Xenotransplantation[▽]

Nicolas C. Issa,[1] Robert A. Wilkinson,[1] Adam Griesemer,[3] David K. C. Cooper,[2] Kazuhiko Yamada,[3] David H. Sachs,[3] and Jay A. Fishman[1]*

- **Quantification requires correction for circulating cells or free DNA** to distinguish "infection" from pig cell carriage (e.g., PCMV or PERV).
  - Pig- MHC-I gene (low copy number)
  - Pig Mitochondrial Cytochrome c oxidase subunit II (high copy number)
- Lack of correlation of viral load with immunosuppression may suggest chimerism or graft injury.

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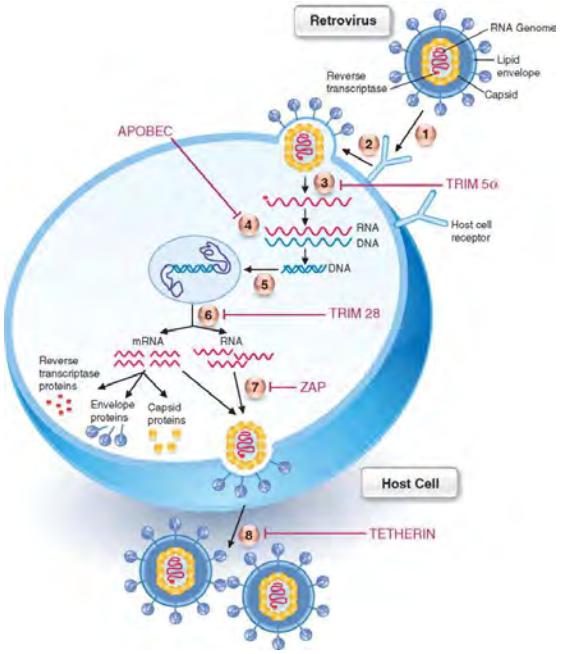

The retroviral life cycle and sites of activity of the major antiviral restriction factors. Envelope glycoproteins of the retrovirus interact with specific host-cell membrane protein receptors. (1) The retroviral envelope fuses with the plasma membrane and enters the host cell. (2) Following fusion, the nucleocapsid enters the cytoplasm and (3) uncoating of viral core occurs. (4) Viral reverse transcriptase copies single strand viral RNA into double-stranded DNA. (5) Viral DNA is transported into the nucleus and integrated into host-cell chromosomal DNA. (6) Integrated viral DNA is transcribed by the host-cell RNA polymerase, generating mRNA molecules and new viral genomic RNA molecules [TRIM28 blocks viral transcription]. Viral mRNAs are translated into viral proteins (envelope, capsid, and reverse transcriptase). (7) Newly synthesized viral proteins and genomic RNA gather to form immature viral particles [ZAP degrades viral RNAs]. (8) New virions bud from the cell surface, acquiring an envelope including host-cellular and viral proteins from the cell membrane [Tetherin traps virions on the cell surface].

Meije Y, Tönjes RR, Fishman JA. Retroviral Restriction Factors and Infectious Risk in Xenotransplantation. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons*. 2010;10(7):1511-1516.

50

Strategies to reduce risk of PERV Transmission?

- Choice of non-transmitting or null animal donor (Garkavenko et al, 2008; Hector et al, 2007)
  - Is it desirable or necessary to have source animals lacking functional PERV-A and -B and without any PERV-C sequences?
- Neutralising antibodies/vaccine development (Fiebig et al 2003)
- Intrinsic antiviral activities
  - APOBEC (Dorrschuck et al 2008; Jonsson et al 2007)
  - Telithrin overexpression reduces PERV release from pig cells in vitro (Mattiuzzo et al, 2010)
- **Antiviral chemotherapies: Multiple classes of agents effective in vitro** (Powell et al 2000; Qari et al 2001; Stephan et al 2001; Wilhem et al 2001; Argaw, T et al. *Xenotransplantation* 2016: 23: 151– 158)
- **Genetically engineered source animals**
  - Intracellularly-expressed single chain antibodies (Dekker et al 2003)
  - shRNA (short hairpin RNA) in vitro (Karlas et al 2004; Miyagawa et al 2005; Dieckhoff et al 2007) and in vivo (Dieckhoff et al 2008; Ramsoondar et al 2009).
  - siRNA to silence PERV production
  - CRISPR-Cas9 engineering -- RNA-programmable nuclease → Advantage to the inducible CRISPR-Cas9 circuit could protect against future PERV infection.

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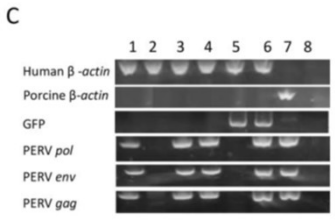
PERV-free Pigs?

- Confirmed that PERVs infect human cells (HEK293T-GFP cells) with horizontal transfer of PERVs among these cells by direct contact.
- Novel PERV junctions produced in the human cell genomes; overrepresented in intra-genic regions and in active chromatin areas.
- CRISPR-Cas9 → inactivated all PERVs in a porcine primary cell line and generated PERV-inactivated pigs via somatic cell nuclear transfer.
- No evident off-site mutations



Detection of human-to-human PERVs transmission in HEK293T cells.

D. Niu *et al.*, *Science* 10.1126/science.aan4187 (2017).

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Amino Acid Sequence of PERV A, C, and AC recombinants

- Over time **individual swine may activate PERV loci and develop new recombinant viruses** (i.e., non-transmitting trait is not permanent).
- **Driven by activity of PERV C genomic locus**
- **Absence of PERV-C in swine suggests no new infectious loci**
- Present assays are cumbersome and inadequate to assess "infectiousness" of a tissue-derived strain (in vitro)

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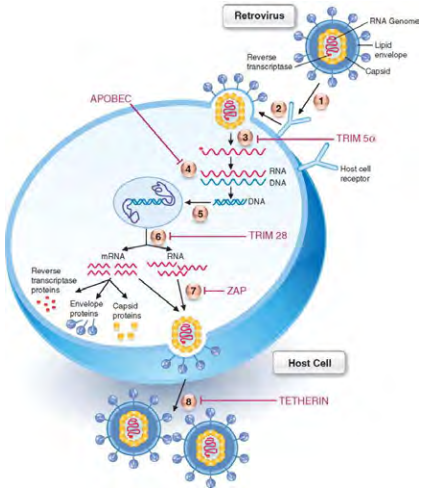

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54

Targeted genome-wide inactivation of porcine endogenous retrovirus activities (PERVs)

Luhan Yang[1,2,3,*,#], Marc Güell[1,2,3,*], Dong Niu[1,4,*], Haydy George[1,*], Emal Lesha[1], Dennis Grishin[1], Weihong Xu[6], Jürgen Poci[1], Ellen Shrock[1], Rebeca Cortazio[1], Robert A Wilkinson[5], Jay A. Fishman[5], George Church[1,2,3,#]

- CRISPR-Cas9 based strategy to inactivate all PERV elements in the porcine genome = 62 copies of PERV elements in the porcine kidney epithelial cell line PK15.
- Using CRISPR-Cas9, we disrupted the catalytic center of the *pol* gene, which catalyzes reverse transcription and is essential for virus replication.
- We isolated cells in which ~100% of the PERV elements had been inactivated and demonstrated a > 1000-fold reduction in transmission of PERVs to human cells, as compared with WT PK15 cells.
- Genome editing demonstrates the possibility of eradicating PERVs *in vitro* for possible application to porcine-to-human xenotransplantation.

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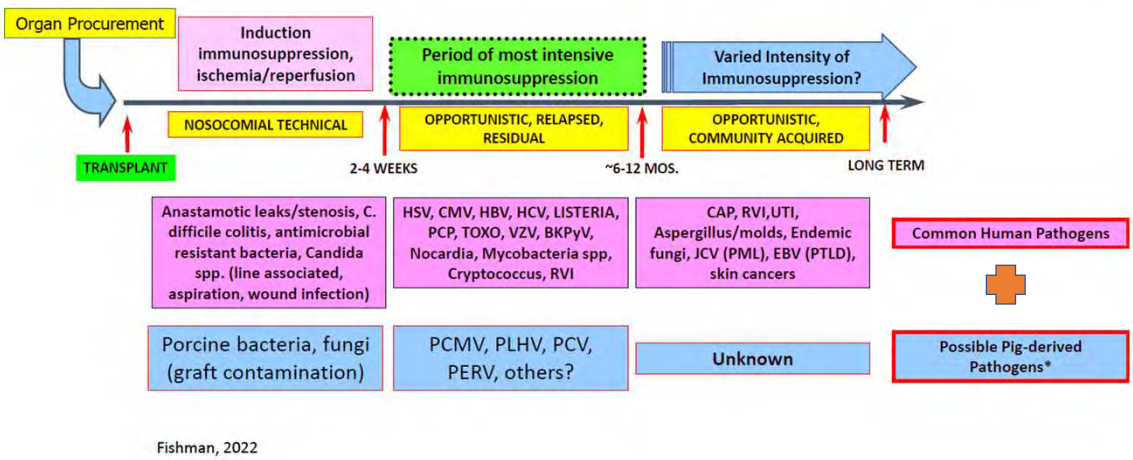
The Timeline of Post-Porcine Xenotransplant Infections



Fishman, 2022

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Deployment of Microbiological Assays in Xenotransplantation				
Assay Type	Screening Source Animals	Xenograft Recipients Monitoring	Xenograft Recipients – Symptomatic Infection or Increased Risk*	Hospital Staff, Healthy Contacts of Recipient
Cultures (Active Infection)	X		X	
Serology (Past Exposures)	X	X	+/-	X
Molecular Assay or Antigen Detection (Active Infection)	X	X	X	+/-
Next Generation Sequencing (Active Infection)		X	X	
Sample Storage	**X**	**X**	**X**	**X**

*Increased risk may be associated with treatment of graft rejection or intercurrent viral infection. Fishman, 2022.

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Infection Control

- Recipient: (T-/B-cell/complement depletion, costimulatory blockade + MMF + steroids?)
  - Universal precautions; vaccinations (Neisseria, H. Flu, COVID, Pneumococcus)
  - Baseline and serial blood (and tissue) samples for common human pathogens and likely pig organisms (not excluded already) – cultures, PCR, metagenomics, histology (PERV, PCMV, PCV, PLHV) – archived cells and sera for nucleic acid and antibody studies.
  - Routine prophylaxis (as for allotransplants)
  - Isolation for readmissions
  - What is not excluded from source herd?
- Surgical and Clinical Facility:
  - Separate from other clinical areas – negative pressure?
- Procurement & Surgical Teams (no known infections in teams studying xeno in primate models)
  - Baseline blood samples (informed consent) stored as cells and sera
  - Blood borne pathogen exposure (fluids) – serial blood samples, consider post-exposure prophylaxis with activity vs PERV (28-day regimen of raltegravir 400mg twice a day with the combination tablet tenofovir DF 300mg/emtricitabine 200mg daily)
- Social contacts of recipient – unknown (baseline samples?)

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Infection Control: Consider …

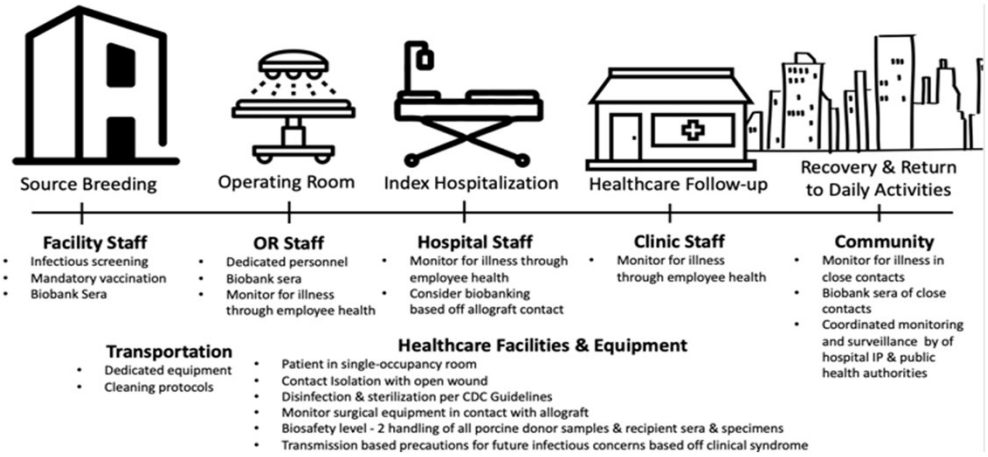

Fig 1. Infection Prevention in Xenotransplant Clinical Trials

From: Nellore, A, Walker, J, Kahn, MJ, Fishman, JA. Moving xenotransplantation from bench to bedside: Managing infectious risk. *Transpl Infect Dis*. 2022;e13909. https://doi.org/10.1111/tid.13909

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Approaching clinical trials

- Each Xenotransplantation Clinical Trial is a "**package**" including:
  Patient Need (Organ) +
  Specific Pig (Breeding/Screening) +
  Immunosuppression (Studied in primate models)
- Likely the infectious risk is not much greater than for allotransplantation – but also not zero.
- Careful screening of source animals is required. Need serologic assays. Have developed quantitative diagnostic methods for some common organisms.
- With immunosuppression, common human pathogens can be expected.
- Decisions regarding PERV's and PCMV in each trial. Some unexpected swine pathogens may be present -- high throughput sequencing tools available.
- Need archiving of donor and recipient specimens for epidemiologic studies
- Advantages: Resistance of porcine xenograft cells to human viruses (HIV, HCV, HBV)
- **Will only understand risk when data from clinical trials emerge.**

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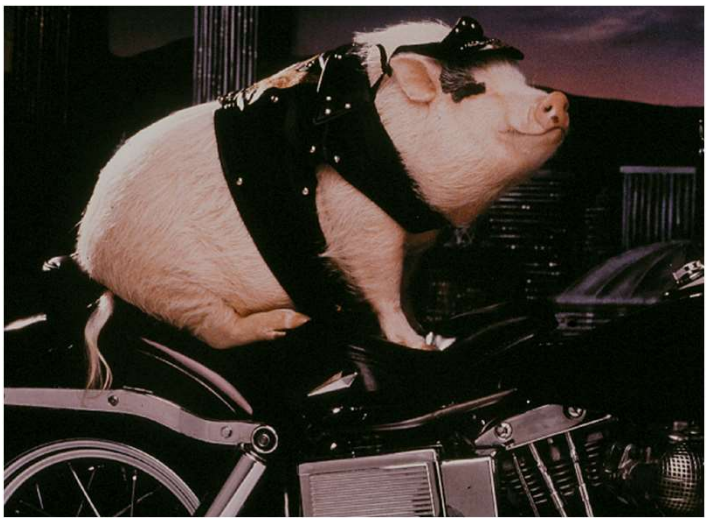

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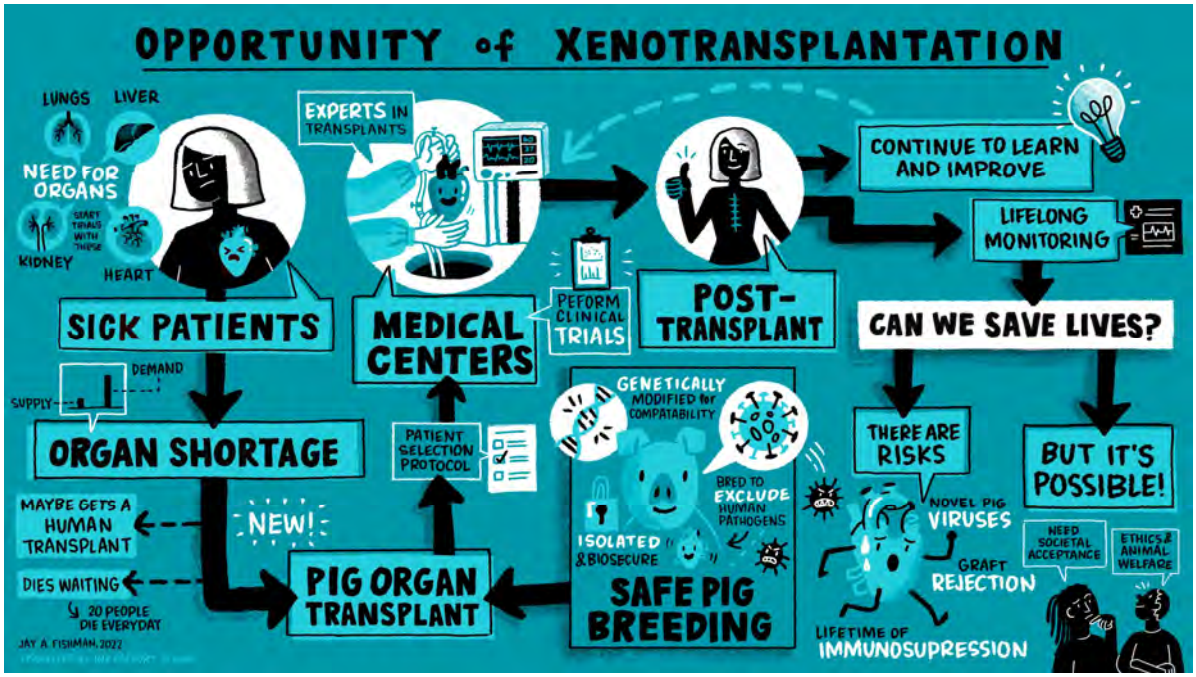

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AST Infectious Disease Community of Practice

NIH National Institute of Allergy and Infectious Diseases

NIH National Heart, Lung, and Blood Institute

- National Institute of Allergy and Infectious Disease: P01AI045897; NIH-NIAID PO1-AI45897; T32: 5T32AI007529-23 (and many others)
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