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Preliminary PCR-based screening indicates a higher incidence of Porcine Endogenous Retrovirus subtype C (PERV-C) in feral versus domestic swine

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Xenotransplantation is considered a potential alternative to allotransplantation to relieve the current shortage of human organs. Due to their similar size and physiology, the organs of pigs are of particular interest for this purpose. Endogenous retroviruses are a result of integration of retroviral genomes into the genome of infected germ cells as DNA proviruses, which are then carried in all cells of the offspring of the organism. Porcine endogenous retroviruses (PERVs) are of special concern because they are found in pig organs and tissues that might otherwise be used for xenotransplantation. PERV proviruses can be induced to replicate and recombine in pigs, and have been shown to infect human cells in vitro. There are three subtypes of PERVs based on differences in the receptor binding domain of the *env* protein; PERV-A, PERV-B, and PERV-C. PERVs A and B can infect human cells in vitro and can recombine with PERV-C, resulting in a recombinant virus with a higher rate of replication in pig and human cell lines. In this study, we used a PCR-based analysis of 50 domestic and 35 feral pigs to study the distribution of PERVs A, B, and C in swine raised under domestic conditions, versus feral swine from rural areas. PERV-A and PERV-B were universal in both domestic and feral swine. Feral swine had a higher incidence of PERV-C (85.7%) compared to domestic swine (42.0%). Further studies in other feral swine herds are ongoing to verify this observation.

Keywords: Porcine endogenous retroviruses, domestic swine, feral swine, PCR analysis

Introduction

Xenotransplantation, the transplantation of living cells, tissues, and organs between species, is a widely suggested alternative to allotransplantation due to the shortage of viable donated organs (Denner and Tönjes 2012; Takeuchi et al. 1998). As of August 2018, there were over 114,000 candidates on transplantation waiting list in the United States, and only approximately 10,100 donors (Organ Procurement and Transplantation Network [https://optn.transplant.hrsa.gov]).

According to the United States Public Health Service, xenotransplantation includes any procedure that involves the transplantation, implantation, or infusion into a human recipient of either 1) live cells, tissues, or organs from a nonhuman animal source, or 2) human body fluids, cells, tissues, or organs that have had *ex vivo* contact with live nonhuman animal cells, tissues or organs (Gola and Mazurek 2014). Although promising, xenotransplantation carries its own challenges and risks that include physiological incompatibilities, immunological rejection, and transmission of infectious agents. Introducing animal tissue and its microbiological flora into the human system and lowering the natural host defense mechanisms for the integration of the organ provides opportunity for transmission of xenogenic infections crossing the species barrier (Brown et al. 1998). Suppressing the

recipient's immune system is a mandatory step in transplantation of organs, to avoid organ rejection. This, however, also makes the recipient more susceptible to infection that might otherwise be easily controlled.

The virulence and clinical outcome of infectious agents are highly unpredictable when they enter a new species and cause infections. Brown et al. (1998) demonstrated this phenomenon in the case of cercopithecine herpes-virus 1 (B virus). In its natural host, the macaque monkey, it causes persistent latent infection with intermittent, recurrent mucocutaneous disease. However in humans, it causes fatal meningoencephalitis (Brown et al. 1998). Because cases of xenographic transmission of infectious diseases in humans have not yet been identified, evidence from human retroviral infections and natural occurring zoonoses like AIDS have been used to assess transplant-related risks of retroviral infections and epidemics in humans (Brown et al. 1998). Human retroviral infections commonly manifest as neurological disorders, immunodeficiencies, and long-latency malignancies for which there are limited treatments available (Gallo 1995; Brown et al. 1998). As such, due to the high risk of retroviral infections, the concerns associated with xenotransplantation are legitimate from a public health perspective.

Retroviruses have an unconventional life cycle compared to other viruses. Their life cycle starts with reverse transcription of the viral RNA genome to DNA, followed by integration of the newly formed DNA into the host genome as a provirus. The provirus is then transcribed to produce the RNA genome and messenger RNA (mRNA). The mRNA directs translation of viral proteins and processing of viral particles, resulting in budding and release of new virions from host cell (Jern and Coffin 2008).

Although retroviruses usually infect somatic cells, occasionally infection of a germline cell by a retrovirus may occur, leading to an integrated provirus passed to the offspring and inherited in Mendelian fashion: this is known as an endogenous retrovirus (ERV) (Jern and Coffin 2008). It has been postulated in some cases that ERVs may provide some evolutionary advantage to the offspring, perhaps allowing the survival of an epidemic of the exogenous form of the virus (Brown et al. 1998). Once integrated into the host genome, these viruses tend to accumulate random mutations with time, eventually leading to an inactive ERV remnant.

ERVs are present in all vertebrate species studied thus far, with a majority of ERVs being inactive. However, of those that are active and replication competent, some have been associated with spontaneous tumors as in endogenous murine leukemia viruses (MLV) and mouse mammary tumor viruses (MMTV) (Frankel et al. 1990; Stoye 2001). Vertebrates have, over time, developed a variety of silencing mechanisms to limit the activity of newly-acquired, replication-competent ERVs. These silencing mechanisms are generally less effective in cases of viruses that have switched hosts (Hayward and Katzourakis 2015) and thus exposure to ERVs from different vertebrate species poses a risk of infections.

Pigs are one of the preferred choices for xenotransplantation because of anatomical and physiological similarities to humans, relatively short generation time, and ease of production of transgenic pigs (Cozzi et al. 2009; Gola and Mazurek 2014). The phylogenetic distance between pigs and humans reduces the risk of transmission of viral infections, with screening and qualified breeding further lowering the risk of other zoonotic infections (Gola and Mazurek 2014). However, the presence of porcine endogenous retroviruses (PERVs) and their capability to produce viral particles, hinders the use of porcine tissue xenografts.

According to the International Committee on the Taxonomy of Viruses (ICTV), PERVs are classified as family: Retroviridae, subfamily:

Orthoretrovirinae, genus: Gammaretrovirus, Porcine type-C oncovirus species (Virus Taxonomy: The 9th Report of the ICTV 2011). Retroviruses have been infecting mammalian species for more than 100 million years according to genomic fossil records and gammaretroviruses as a group have jumped between species frequently (Hayward and Katzourakis 2015). There are three replication competent subtypes of PERVs: PERV-A, PERV-B, and PERV-C, which are identified based on differences in the receptor binding domain of the *env* protein. PERVs A and B are present in the genomes of all pigs and are able to infect human cells *in vitro,* as well as cells of other species (Wilson et al. 1998; Denner and Tönjes 2012). PERV-C is integrated into the genomes of many, but not all, pigs and is restricted to infecting pig cells (Takeuchi et al. 1998; Denner 2016).

The origin of PERVs was most likely a murine retrovirus (Denner and Tönjes 2012). Due to the high homology of PERVs to ape and murine leukemia viruses, researchers have suggested PERVs may be capable of inducing leukemia in a receptive host (Boneva et al. 2001). It should be noted, to date no evidence of human infections due to PERVs has been documented in patients exposed to pig tissue (Boneva et al. 2001).

Numerous transgenic pigs have been generated to produce organs that are more readily accepted by the human immune system, however, it is not currently possible to use genetically engineered pigs for xenotransplants due to lack of knowledge about the role of PERVs, as well as high variability and copy numbers of PERVs in porcine tissue. Recently, Niu et al. (2017) produced PERV-inactivated live pigs from PERV-inactivated primary porcine cell lines by using a combination of CRISPR-Cas9, apoptosis inhibitors, and growth factors. Long-term studies on these PERV-inactivated pigs are being conducted to assess the effects of the absence of PERVs on the hosts (Niu et al. 2017). Currently, the use of these PERV-inactivated pigs is limited to research, and are not approved for human trials.

Currently, there is limited information about the evolutionary history, distribution patterns, roles and potential infectious capability of PERVs. A better understanding of PERVs is essential to prevent the possible emergence of novel xenozoonoses from pig to human transplantations. The purpose of this research was to study and compare the distribution of the three types of PERVs in feral and domestic varieties of pigs. Although with proper containment of domestic herds, the likelihood of PERV transmission from a feral pig into a domestic herd is low, it is nonetheless possible.

Materials and Methods

Sample collection: The total sample size for this study was 85 samples. Tails from 50 domestic piglets were collected on four separate occasions from animals housed in the Fort Hays State University swine operation, these samples being pooled and labeled as Domestic 1 through Domestic 4. Samples from 35 feral swine were obtained by local hunters near the towns Trenton and Ravenna in rural Fannin County, Texas. These samples were pooled and labeled as Feral. The docked tail samples were placed in 95% ethanol and stored at -20° C until DNA was extracted.

DNA extraction and isolation: Before performing extraction, the tissue samples were washed with distilled water. Genomic DNA was extracted using Qiagen DNeasy Blood and Tissue extraction kit (Hilden, Germany) following the manufacturer's instructions and eluted in 50 µl of nuclease-free water. The extracted DNA was visualized in 1% agarose gel and quantified using a spectrophotometer (Nanodrop Technologies, Wilmington, DE). Following isolation, DNA samples were stored at -20°C until further analysis.

Table 1. PERV-specific primers used for detection of three distinct variants of the *env* gene of PERVs A, B, and C from domestic and feral swine tissue (Liu et al 2011).

PERV Subtype	Sequence	Amplicon size (bp)
PERV-A	F:5′-TGGAAAGATTGGCAACAGCG-3′ R:5′-AGTGATGTTAGGCTCAGTGG-3′	359
PERV-B	F: 5′-TTCTCCTTTGTCAATTCCGG-3′ R:5′-TACTTTATCGGGTCCCACTG-3′	263
PERV-C	F:5′-CTGACCTGGATTAGAACTGG-3′ R:5′-ATGTTAGAGGATGGTCCTGG-3′	281

Amplification and analysis: Polymerase Chain Reaction (PCR) was used to detect presence of PERV genomes in samples of porcine genomic DNA. Primer sets and PCR cycling conditions were as described by Liu et al. (2011). Three primer sets (Table 1) which amplify a small region of the *env* gene (Sigma-Aldrich, St. Louis, MO) were used to detect the three PERV subtypes. PCR was conducted using Phusion High-Fidelity Polymerase kit from New England Biolabs (Ipswich, MA).

PCR reactions were conducted in 50 µl of reaction mixture: 10 µl of 5X Phusion High Fidelity Buffer, 2.5 µl of 10 µM PERV-A, PERV-B, or PERV-C forward primer, 2.5 µl of 10 µM of reverse primers, 1 µl of 10 mM dNTPs, 0.5 µl Phusion Polymerase, 2 µl of template DNA and 31.5 µl of nuclease free water. PCR reaction conditions were as follows: 95°C for 5 minutes for initial denaturation, 95°C for 30 seconds, 55°C for 45 seconds, 72°C for 1 minute, repeated for 30 cycles with final extension at 72°C for 7 minutes. Amplicons were visualized by 2% agarose gel (Agarose low EEO, Thermo Fisher Scientific, Waltham, MA) electrophoresis in TAE and stained with SYBR safe DNA gel stain (Thermo Fisher Scientific, Waltham, MA). A Promega 1 kb DNA ladder (Madison, WI, USA) was used to estimate the sizes of the amplicons. The PCR amplicons were visualized in a Kodak Gel Logic 100 Imaging System (Rochester, New York).

Results

PERV detection in domestic samples: DNA extracted from 50 tissue samples from domestic pigs was screened by PCR for detection of *env* gene sequence for PERV-A, PERV-B, and PERV-C. PERV-A and PERV-B were ubiquitous in all the samples, whereas PERV-C was present in 21 out of 50 (42%) samples (Table 2). Representative examples of the PCR amplicons for PERVs A, B, and C from the domestic pigs are shown in Figure 1.

PERV detection in feral samples: DNA extracted from 35 tissue samples from feral pigs were screened by PCR process using the same primers for PERV-A, PERV-B,

Table 2. Results of PCR analysis with PERV-specific primers of domestic swine from the FHSU farm and feral swine collected by hunters in rural Fannin County, Texas.

Sample group	Sample size	PERV-A		PERV-B		PERV-C	
		Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Domestic 1	21	21 (100)	0 (0)	21 (100)	0 (0)	6 (28.6)	15 (71.4)
Domestic 2	12	12 (100)	0 (0)	12 (100)	0 (0)	7 (58.3)	5 (41.7)
Domestic 3	7	7 (100)	0 (0)	7 (100)	0 (0)	5 (71.4)	2 (28.6)
Domestic 4	10	10 (100)	0 (0)	10 (100)	0 (0)	3 (30)	7 (70)
Feral	35	35 (100)	0 (0)	35 (100)	0 (0)	30 (85.7)	5 (14.3)

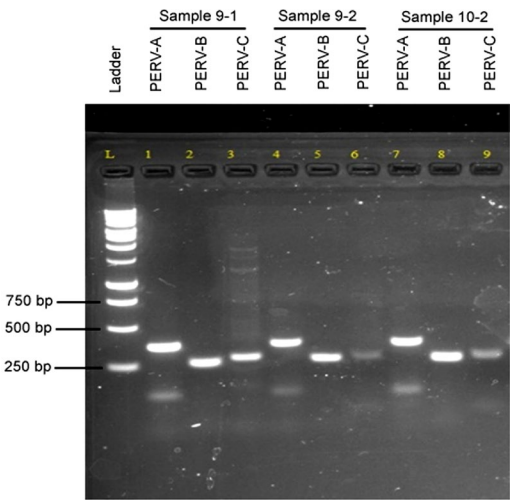

Figure 1. Examples of PERVs A, B, and C *env* amplicons from domestic porcine samples using PCR primers from Liu et al (2011). In these examples all three PERV subtypes are present.

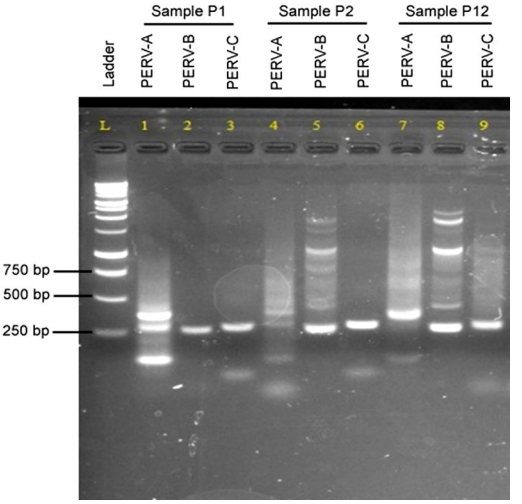

Figure 2. Examples of PERVs A, B, and C *env* amplicons from feral pigs using PCR primers from Liu et al (2011). In these examples all three PERV subtypes are present.

and PERV-C. In these samples PERV-A and PERV-B were universal and PERV-C was detected in 30 of 35 (85.7%) samples (Table 2). Representative examples of PCR amplicons for PERVs A, B, and C from the feral pigs are presented in Figure 2.

Discussion

Our data showed the presence of PERVs A and B in all of the porcine tissue tested, both from domestic and feral sources. This observation is consistent with the findings of other researchers in that PERV-A and PERV-B are present in all pigs (Denner et al. 2009; Takeuchi et al. 1998; Patience et al. 1997). Our data also was consistent with the observations of other workers in that PERV-C is not present in all pigs. Interestingly, our data indicated PERV-C was present in a greater number of feral pigs (85.7%) than in domestic pigs (42%). We hypothesize that this may be due to the carefully managed breeding programs used by most domestic swine producers, compared to the uncontrolled mating and reproduction that occurs in feral swine populations.

Since PERVs A and B are carried and passed in a Mendelian fashion by all pigs, it is not possible to produce animals free of these viruses by selective breeding programs alone. While PERV-C is only carried by some pigs, it should be possible, through prior testing before mating, to produce swine herds that are entirely free of PERV-C. The production of herds free of PERV-C should negate the likelihood of producing potentially harmful recombinant PERV-A/C or PERV-B/C proviruses, which could be detrimental in xenotransplantation and other applications.

PERVs are thought to have originated in African members of the *Suidae* family about 7.5 million years ago. However, PERV-C originated nearly 3.5 million years later than PERV-A and PERV-B, likely due to a recombination event between PERV-A and an unknown ancestor (Niebert and Tönjes 2005). The later introduction of PERV-C into pigs is thought to be the reason for lack of the universal presence of PERV-C in pigs. (Wood et al. 2009).

The role of ERVs in various animals is largely unknown, except in a few cases, such as sheep (*Ovis aries*), where they have proven to be

beneficial. In sheep, an ERV has been shown to be instrumental in the formation of the placenta (Dunlap et al. 2006). In other cases, the presence of ERVs can also be detrimental and are found to be correlated with cancers, germ-line mutations, autoimmune disorders, and replication-competent viral particles (Mager and Stoye 2014). An active retrovirus infection and endogenization process is now occurring in koalas (*Phascolarctos cinereus*) in Australia (Tarlington et al. 2006), and provides an opportunity to study and possibly gain invaluable insights into retroviral endogenization (Stoye 2006). The koala retrovirus (KoRV), thought to have been transmitted from an unknown rodent to koalas, is actively spreading among these animals. KoRV has been associated with myeloid leukemias, neurodegenerative diseases, immunodeficiencies, and/or lymphomas in koalas (Denner 2007). KoRV has endogenized into the germ line of some koalas, but retains characteristics of exogenous retroviruses in other populations (Kinney et al. 2016). Similarly, our observation of a greater carriage of PERV-C among feral swine populations could indicate the beginning of the spread of PERV-C among these animals. If this is the case, given sufficient time, PERV-C could become ubiquitous in feral swine herds within a particular location. We are conducting further work to assess whether there is indeed a greater carriage of PERV-C among other feral swine herds in general, or if our results represent an isolated incident.

Conclusion

Currently, the main strategy for control of PERVs in pigs is selective breeding and subsequent genetic modification to possibly rear PERV-free animals. To further progress in the field of pig-to-human xenotransplantation, it is imperative to understand the characteristics, distribution and evolutionary history of PERVs. Thus, studies in the distribution of PERVs in select breeds can contribute to establishing patterns of inheritance of PERVs in future generations. Although, there has been significant interest in PERV related research, there is still much left to be discovered about PERVs. Hence, further study and development of sensitive methods for detection of PERVs is essential for elimination, or at the least, controlling the risk of PERV related zoonoses.

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Use of Animals

Tissue collection protocols were approved by the Fort Hays State University Institutional Animal Care and Use Committee (IACUC protocol 17-0004EX).

Literature Cited

Boneva. R.S., Folks, T.M. and Chapman, L.E., 2001. Infectious Disease Issues in Xenotransplantation. Clinical Microbiology Reviews 14:1-14.

Brown, J., Matthews, A.L., Sandstrom, P.A. and Chapman, E.L. 1998. Xenotransplantation and the risk of retroviral zoonosis. Trends in Microbiology 6:411-415.

Cozzi, E., Bosio, E., Seveso, M., Rubello, D. and Ancona, E. 2009. Xenotransplantation as a model of integrated, multidisciplinary research. Organogenesis 5:288-296.

Denner, J. 2016. How Active Are Porcine Endogenous Retroviruses (PERVs)? Viruses 8:215.

Denner, J., Schuurman, H. and Patience, C. 2009. The International Xenotransplantation Association consensus statement on conditions for undertaking clinical trials of porcine islet products in type 1 diabetes. Chapter 5. Strategies to prevent transmission of porcine endogenous retroviruses. Xenotransplantation 16:239–248.

Denner, J. 2007. Transspecies transmissions of retroviruses: New cases. Virology 369:229-233.

Denner, J. and Tönjes, R.R. 2012. Infection barriers to successful xenotransplantation focusing on porcine endogenous retroviruses. Clinical Microbiology Reviews 25:318–343.

Dunlap, K.A., Palmarini, M., Varela, M., Burghardt, R.C., Hayashi, K., Farmer, J.L. and Spencer, T.E. 2006. Endogenous retroviruses regulate periimplantation placental growth and differentiation. Proceedings of the National Academy of Sciences of the United States of America 103:14390-14395.

Frankel, W.N., Stoye, J.P., Taylor, B.A. and Coffin, J.M. 1991. A genetic linkage map of endogenous murine leukemia viruses. Genetics 124:221-236.

Gallo, R.C. 1995. Human Retroviruses in the second decade: A personal perspective. Nature Medicine 1:753-759.

Gola, J. and Mazurek, U. 2014. Detection of porcine endogenous retrovirus in xenotransplantation. Reproductive biology 14:68-73.

Hayward, A. and Katzourakis, A. 2015. Endogenous Retroviruses. Current Biology 25:R644-R646.

Jern, P., and Coffin, J.M. 2008. Effects of Retroviruses on Host Genome Function: Annual Review of Genetics 42:709–732.

Kinney, M.E. and Pye, G.W., 2016. Koala retrovirus: a review. Journal of Zoo and Wildlife Medicine 47:387–396

Lee, D., Kim, N.Y., Bae, G.E., Lee, H.J., Kwon, M., Kim, S.S., Lee, H.T., Yang, J.M. and Kim, Y.B. 2008. Transmissible infection of human 293T cells with porcine endogenous retroviruses subgroup a from NIH-miniature pig. Transplantation Proceedings 40:3742–3745.

Liu, G., Li, Z., Pan, M., Ge, M., Wang, Y. and Gao, Y. 2011. Genetic prevalence of porcine endogenous retrovirus in Chinese experimental miniature pigs. Transplant Proceedings 43:2762–2769.

Mager, D.L. and Stoye, J.P. 2014. Mammalian Endogenous Retroviruses. Microbiology Spectrum 3:MDNA3-0009-2014.

Niebert, M. and Tönjes, R.R. 2005. Evolutionary Spread and Recombination of Porcine Endogenous Retroviruses in the *suiformes*. Journal of Virology 79:649–654.

Niu, D., Wei, H.-J., Lin, L., George, H., Wang, T. and Lee, I.-H. 2017. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. Science 357:1303–1307.

Organ Procurement and Transplantation Network [https://optn.transplant.hrsa.gov].

Patience, C., Takeuchi. Y. and Weiss, R.A. 1997. Infection of human cells by an endogenous retrovirus of pigs. Nature Medicine 3:282-286.

Stoye, J.P. 2001. Endogenous retroviruses: Still active after all these years? Current Biology 11:R914–R916.

Stoye, J.P. 2006. Koala retrovirus: a genome invasion in real time. Genome Biology, 7:241.

Takeuchi, Y., Patience, C., Magre, S., Weiss, R.A., Banarjee, P.T., Tisser, P.L. and Stoye, J.P. 1998. Host Range and Interference Studies of Three Classes of Pig Endogenous Retrovirus. Journal of Virology 72:9986-9991.

Tarlington, R.E., Meers, J. and Young, .P.R. 2006. Retroviral invasion of the koala genome. Nature 442:79-81

Wilson, C.A., Wong, S., Muller, J., Davidson, C.E., Rose, T.M. and Burd, P. 1998. Type C Retrovirus Released from Porcine Primary Peripheral Blood Mononuclear Cells Infects Human Cells. Journal of Virology 72:3082-3087.

Wood, A., Webb, B.L.J., Bartosch, B., Schaller, T., Takeuchi, Y. and Towers, G.J. 2009. Porcine endogenous retroviruses PERV A and A/C recombinant are insensitive to a range of divergent mammalian TRIM5α proteins including human TRIM5α. The Journal of General Virology 90:702-709.

Virus Taxonomy: The 9th Report of the ICTV (2011). Retroviridae. [https://talk.ictvonline.org/ictvreports/ictv_9th_report/reverse-transcribing-dna-and-rna-viruses-2011/w/rt_viruses/162/retroviridae-figures]