Winter 2018

Distribution Of Porcine Endogenous Retrovirus (PERV) Variants In Domestic And Feral Pig

Rashmi Acharya
Fort Hays State University, r_acharya@mail.fhsu.edu

DISTRIBUTION OF PORCINE ENDOGENOUS RETROVIRUS (PERV)

VARIANTS IN DOMESTIC AND FERAL PIGS

being

A Thesis Presented to the Graduate Faculty

of the Fort Hays State University in

Partial Fulfillment of the Requirements for

the Degree of Master of Science

by

Rashmi Acharya

B.Sc., Tribhuvan University

Date: _____ Approved: _____

Major Advisor

Approved: _____

Chair, Graduate Council

This thesis is for

the Master of Science Degree

by

Rashmi Acharya

has been approved by:

_____

Chair, Supervisory Committee

_____

Supervisory Committee

_____

Supervisory Committee

_____

Supervisory Committee

_____

Chair, Department of Biological Sciences

ABSTRACT

Xenotransplantation is considered an 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 copies (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 tissue that might be used for xenotransplantation.

PERV proviruses, already incorporated into the pig's genome, can be induced to replicate and recombine in pigs, and have been shown to infect human cells in vitro. There are three classes of PERVs, namely PERV-A, PERV-B, and PERV-C. PERV-A and PERV-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, a PCR based analysis of 50 domestic and 35 feral pigs was carried out to study the distribution of PERVs A, B, and C. PERV-A and PERV-B were universal in both domestic and feral pigs. The feral varieties of pigs displayed a higher frequency of 85.67% of PERV-C compared to 42.00% in domestic pigs. However, comparative study of presence of PERVs A, B, and C in different breeds of domestic pigs shows there is variation in distribution among breeds, and among individuals of same breeds. From the results of this study, I hypothesize that presence of endogenized PERV genomes in individuals of the same breed is dependent on genetic properties of individual pigs.

ACKNOWLEDGEMENTS

I am immensely grateful to numerous people who helped me throughout my time in graduate school, without whom this work would not have been possible. Firstly, I express my deepest gratitude to my advisor Dr. Eric Gillock for accepting me into his lab and giving me the opportunity to learn and grow under his guidance. My time under Dr. Gillock's supervision has not only helped me become a better scientist but also a better person. I would also like to thank my graduate committee members Dr. James Balthazor, Dr. Brian Maricle, and Dr. William Stark for their counsel and support throughout my program.

I thank the Fort Hays State University Department of Biological Sciences and the Graduate School. I also extend my gratitude to all my peers who made my time here very enjoyable. I am very appreciative for the support provided by Ms. Anuja Paudyal in providing instructions on lab techniques and answering my queries related to various topics.

I am indebted to Dr. Terry Balthazor and the Balthazor Fellowship that supported me through the second year of my studies. It lifted a great burden from me and allowed me to dedicate myself to my research. Funding for this research was provided by a K-INBRE student-Faculty Mini-Grant, 2017-2018 academic year. This project was supported by the National Institute of General Medical Sciences (P20 GM103418) from the National Institute of Health.

It would not have been possible for me to achieve this feat without the outpouring of love and support extended by my friends and family. A special thank you will always be

present for my close friend Ms. Urmila Kandel in all my accomplishments, for being a pillar of support for me night and day throughout all these years from middle school to a graduate degree. I am eternally grateful to the Bhakta family for accepting me as their own and giving me a huge loving family and a place to call home.

The most important mention goes to my family. Whatever I am today, I am only because of my family. I am continually awe-inspired by my mother's exemplary work ethic, fierce independence, and her unbridled love for her children. I am blessed to have a father who has always had unwavering confidence in me, at times more so than I've had in myself. My brother, my constant companion, my best friend who has been my biggest motivator to reach my goals and keep setting new ones. I dedicate this work to my dear mother Binu Acharya, my wonderful father Ram Prasad Acharya, and my loving brother Rikesh Acharya.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF APPENDICES

Page

PREFACE

This thesis follows the style of Transactions of Kansas Academy of Science.

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 (a) live cells, tissues, or organs from a nonhuman animal source, or (b) 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 recipient's immunological barrier is a mandatory step in transplantation of organs, to avoid organ rejection. This, however, also makes the recipient more susceptible to infection that would normally be controlled by their immune system.

The virulence and clinical outcome of infectious agents are highly unpredictable when they enter a new species and cause infections. Brown et al. (1998) exemplified the case of cercopithecine herpes-virus 1 (B virus), which in its natural host macaque monkey 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, long-latency malignancies, with long periods of clinical latency and 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.

Endogenous Retroviruses

A retrovirus is an encapsulated dimer of positive-sense single-stranded RNA, which in turn is enclosed in a lipid bilayer envelope (Figure 1). Retroviruses have an unconventional life cycle compared to other viruses. Their life cycle starts with reverse transcription of the viral genome from RNA to DNA, followed by integration of the newly formed DNA into the host genome, resulting in the provirus. The provirus is then transcribed to form the RNA genome and messenger RNA (mRNA). The mRNA directs

translation of viral proteins and processing of viral particles, resulting in budding of new virions from host cell surface (Jern and Coffin 2008).

Although retroviruses usually infect somatic cells, occasionally infection of a germline cell by a retrovirus may lead to an integrated provirus that is 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 this process may have provided some evolutionary advantage to the animal, perhaps in surviving an ongoing epidemic of the exogenous form of the virus (Brown et al. 1998). Once integrated into the host genome, these viruses accumulated random mutations along with the cellular genes of the animals and with time attained a dormant stage.

ERVs are present in all vertebrate species studied thus far, and a majority of ERVs are 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.

Porcine Endogenous Retroviruses

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 and screening and qualified breeding further lowers the risk of other zoonotic infections (Gola and Mazurek 2014). However, the presence of PERVs and their capability to transcribe viral particles hinders the use of porcine 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 (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, identified based on variation of the *env* gene. PERV-A and PERV-B are present in the genomes of all pig strains, at different copy numbers, and are polytropic, which means they are able to infect human cells *in vitro* (Figure 2) (Wilson et al. 1998; Denner and Tönjes 2012) and cells of other species. PERV-C is integrated into the genome in many, but not all, pigs and is ecotropic, meaning they are restricted to infecting pig cells (Takeuchi et al. 1998; Denner 2016).

The genomes of replication-competent PERVs are encoded by RNA, which is then transcribed into proviral DNA by the viral enzyme reverse transcriptase (RT) (The 9th Report of the ICTV 2011). Viral particles are assembled at the cellular membrane, composed of lipids and protein derived from host cell, and are released by a budding

process. The length of the provirus is about 9000 base pairs (bp) and contains coding sequences *gag* (group-specific antigen), *pol* (polymerase gene), and *env* (envelope gene) (Figure 3) (Łopata et al. 2018). At the proviral stage, these genes are bounded by non-coding terminal regions known as long terminal repeats (LTR), which contain promoter, enhancer, and regulatory elements (Kimsa et al. 2014). The *gag* genes encode the structural proteins of the matrix (MA), the capsid protein (CA) which is the main structural protein, and the nucleocapsid (NC).

In gammaretroviruses, there is an additional protein localized in the *Gag* polyprotein between MA and CA, the p12 protein, which contributes in the integration of the double-stranded DNA (dsDNA) within the genome of the host cell, as well as in the release of new virus particles (Łopata et al. 2018). The *pol* genes code for the RT, the integrase (IN), and protease (PR) that participate in the transcription process and integration of the viral DNA copy into the host genome (Denner and Tönjes 2012). The *env* gene encodes a precursor molecule which is cleaved by a cellular furin-like protease into two envelope protein components: the surface envelope protein SU (gp70) and the transmembrane envelope protein TM (P15E) (Denner and Tönjes 2012; Łopata et al. 2018). The TM protein, which is buried in the lipid bilayer, mediates the membrane fusion reactions and anchors the SU protein to the surface of viral particles. The SU protein is responsible for binding with the host receptor (Łopata et al. 2018). Comparisons of the TM protein of PERV-A and PERV-B have shown 92% amino acid identity to one another and 63 to 66% identity to the corresponding region from gibbon ape leukemia virus (GALV), feline leukemia virus (FLV), and friend virus (FV) strain of MLV.

The origin of PERVs was most likely a murine retrovirus (Denner and Tönjes 2012). Both viruses are phylogenetically related (Figure 4) (Klymiuk et al. 2002). Sequence alignment of the IN protein shows 79% identity between PERVs and MLV, and they show similarities in genomic integration target site selection, preferentially integrating near transcription start sites (Moalic et al. 2006). Due to the high homology of PERVs to ape and murine leukemia viruses, researchers have suggested PERV may be capable of inducing leukemia in a receptive host (Boneva et al. 2001). To date, no evidence of human infection with PERV has been documented in patients exposed to pig tissue, in spite of the presence of long-term PERV microchimerism (stable presence of a minority of non-self-cells in a host) in some patients (Boneva et al. 2001).

Numerous transgenic pigs have been generated to produce organs that are more readily accepted by the human body, however, it is not currently possible to use genetically engineered pigs for xenotransplants due to lack of knowledge about the role of PERVs, high variability, and copy numbers of PERV genomes in pigs. Niu et al. (2017) have been able to produce PERV-inactivated live pigs from PERV-inactivated primary porcine cell lines obtained by using a combination of CRISPR-Cas9, apoptosis inhibitor, and growth factors. Long-term studies on these PERV inactivated pigs are being conducted to collect information on the functionalities of PERVs in relation to the hosts (Niu et al. 2017). However, use of these PERV-inactivated pigs is limited to research.

Currently, there is very limited information about the evolutionary history, distribution patterns, roles and potential infectious capability of PERVs. A better understanding of

PERVs in 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 three types of PERVs in feral and domestic varieties of pigs and to establish whether a pattern exists among various breeds. It was hypothesized that if there was a relationship between PERV distribution and breeds of pigs, then the distribution of PERVs would be universal in individuals of the same breed.

MATERIALS AND METHOD

Sample collection:

The total sample size for this study was 85 samples. Tails from 50 domestic piglets of four breeds, namely Berkshire, Hampshire, Yorkshire, Blue Butt Cross (blue spotted cross from a Hampshire X Yorkshire) were collected from Fort Hays State University (FHSU) farm (Table 1). Samples from 35 feral pigs of unknown breeds were obtained from towns Trenton and Ravenna in Texas (Figure 5). The docked tail samples were placed in 95% ethanol and stored in -20° C freezer 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 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.

Amplification and analysis:

Polymerase Chain Reaction (PCR) was used to detect presence of PERV genomes in samples of pig genomic DNA. Primer sets and PCR cycling conditions followed Liu et al. (2011). Three primer sets (Table 2) which amplify a small region of the *env* gene (Sigma-Aldrich, St. Louis, MO) were used to individually detect three PERV genotypes. PCR

was conducted using Phusion High-Fidelity Polymerase kit from New England Biolabs (Ipswich, MA).

PCR reaction was 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 with TAE (i.e., Tris-acetate-EDTA) and SYBER safe DNA gel stain (Thermo Fisher Scientific, Waltham, MA, USA). A 1 kb DNA ladder (Promega, Madison, WI, USA) was used to estimate the sizes of the amplicons.

After electrophoresis, the gel was transferred to a Kodak Gel Logic 100 Imaging System to visualize bands of amplified DNA fragments. Resulting DNA bands were compared to detect presence or absence of the three variations of PERV genomes and their occurrence in domestic and feral varieties of pigs. Amplified PCR products were purified using QIAquick PCR purification Kit (Qiagen, Hilden, Germany) following manufacturer's instructions and eluted in nuclease-free water. Samples were prepared at a concentration of 2 ng μL^{-1} x kb with a total volume of 10 µL for sequencing, and sent to Genewiz (South Plainsfield, New Jersey, USA). Primer sets were sent with samples according to instructions from Genewiz.

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. Representative examples of the PCR amplicons for PERVs A, B, and C from the domestic pigs are shown in Figure 6. For these samples, the breeds of the pigs were known, hence, the distribution of PERVs among different breeds (Table 1) was analyzed and the results are demonstrated in Table 3.

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, and PERV-C. In these samples PERV-A and PERV-B were universal and PERV-C was detected in 30 samples out of 35 (85.7%). Some examples of PCR amplicons for PERVs A, B, and C from the feral pigs are presented in Figure 7.

Sequencing results:

The sequencing attempt failed because the PCR primers were not suitable.

DISCUSSION

We have described a PCR-based analysis for detection of PERV sequences in pigs of various breeds. This experiment was designed to detect the frequency of PERVs, compare their distribution in breeds of domestic pigs against feral pigs. The gene targeted in this experiment was *env* gene, which encodes viral envelope proteins, and partially determines the host tropism of the virus. There was a higher presence of PERV-C in feral herds (85.7%) compared to domestic breeds (42%) (Figure 7). This supports the hypothesis that frequency of PERV-C is lower in domestic pigs than feral pigs. These results favor the idea of selective breeding of herds with low copy number of PERVs in their genomes, to avoid transmission and recombination events.

Variation in the PERV-C positive samples in the domestic pigs and among individual pigs of the same breed demonstrated that presence of PERV-C proviral DNA is variable in the same breed. If distribution of PERV-C was breed specific then PERV-C would have been present in all pigs belonging to the same breed. However, absence of PERV-C in some individuals and presence in other individuals of same breed show that distribution of PERV-C is not breed specific and is not as widespread as PERV-A and PERV-B (Figure 8). My results suggest that distribution of PERV-C does not depend on presence of PERV-C in a breed but depends on individual genetic characteristics of each pig. My data are consistent with the findings that PERV-A and PERV-B are present in all pigs (Denner 2009; Takeuchi et al. 1998; Patience et al. 1997) (Table 3, Table 4) and so the breeding of completely PERV free pigs is not possible. However, PERV-C is not ubiquitous so breeding of herds free of PERV-C is possible.

Sequencing of amplified PCR products might have provided more insights as to causes of variance in distribution of PERVs in domestic and feral pigs, however due to failure in sequencing, I do not have the data to make any inferences. Occasionally, primers suitable for PCR amplification, might not be compatible to use as sequencing primers because PCR is an exponential process, and as such, even if there is only a small proportion of the target DNA present, it can be amplified in multiple cycles to produce good PCR amplification results. However, sequencing is a linear process so the target DNA cannot be sequenced with the same primer in cases if the primer is inefficient (Biomedical Research Core Facilities [https://brcf.medicine.umich.edu/cores/dna-sequencing/faqs/sanger-sequencing-faqs/]). Inefficiency in priming also could be due to reasons intrinsic to structure or sequence of primers, primers forming dimer or hairpin, effect of folded structures of DNA template, or any combination of these factors (Genewiz, Diagnosing Sanger).

Denner et al. (2009) found PERV-A/C recombinants integrated in DNA of somatic pig cells, but not in the pig germ line. PERV-A/C recombinant viral particles showed a higher replication rate than normal PERV variants in human cells after adaptation to human cells which may be associated with higher pathogenicity (Denner and Tönjes 2012). A recombinant virus which carries host cell receptor-binding region of human cells, from PERV-A, and the rest of the recombinant genome from PERV-C has a 500 fold increased infectivity than normal PERV-A variant (Harrison et al. 2004). Kimsa et al. (2014), suggested that a lack of PERV-C active loci could reduce the chances of PERV-A/C recombination. To decrease the likelihood of a recombinant PERV-A/C

provirus, measures can be applied for selectivity of pigs with low copy numbers of PERV-A, PERV-B, and free of PERV-C. In cases of such selective breeding, screening of each individual pig in multiple stages of growth is necessary for close monitoring of PERV-C presence. Long- term studies by Niu et al. (2017), on PERV-inactivated pigs produced by applying CRISPR-Cas9, technology might be able to provide insight into the role of PERVs in pigs and thus lead to development of novel practices in breeding and production of PERV free pigs in the future.

PERV 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, due to a recombination event between PERV-A and an unknown ancestor (Niebert and Tönjes 2005). This could be the reasoning behind the lack of universal presence of PERV-C, unlike other PERV variants. The study was unable to determine if the origin of PERV-A and PERV-B was independent integration into the pig genome or due to some recombinant event between ancient variants similar to the origin of PERV-C (Niebert and Tönjes 2005). Due to the shorter presence of PERV-C in pig genomes, PERV-C might not have been subjected to enough mutations to lower its RT activity, and as a result is more prone to recombination events (Wood et al. 2009). The role of ERVs in various animals is largely unknown, except in a few animals like sheep (*Ovis aries*). In some cases, it has proven to be beneficial for the animals. This is the case in sheep where an ERV leads to the formation of the placenta (Dunlap et al. 2006). In most hosts ERVs are detrimental, found to be in relation with cancers, germ-line mutations, autoimmune disorders, and replication-competent viral particles (Mager and Stoye 2014). A recent

example is the case of transmission of retroviruses similar to PERV, the koala retrovirus (KoRV) from unknown rodents to koalas (*Phascolarctos cinereus*). KoRV has been associated with myeloid leukemias, neurogenerative 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 others (Kinney et al. 2016). An active infection and endogenization process is now occurring in Koalas (Tarlington et al. 2006), which gives us an opportunity to study and possibly gain invaluable insights into retroviral endogenization (Stoye 2006).

Screening of xenograft tissues and organs is a crucial step in determining the viability of porcine tissue for xenotransplantation. Along with screening for other infectious agents like the influenza virus, *Listeria monocytogenes*, *Yersinia* species, porcine cytomegalovirus, porcine gammalymphotropic herpesvirus, and swine torque virus, screening for PERVs is also a major step in ensuring the safety of such procedures (Boneva et al. 2001; Denner and Tönjes 2012). At present, the main strategy for prevention against transmission of porcine microorganisms is selective breeding of donor pigs.

In 1997, amidst a putative spread of infectious diseases via pig-to-human transplant and inability to assess the risks, the FDA placed a hold on ongoing clinical trials involving new drug developments and cellular transplants, pending the development and implementation of monitoring strategies (Denner and Tönjes 2012). In recent years, research has shown PERVs can replicate and recombine in pigs and although there has been no evidence of PERV infection of humans *in vivo*, PERVs have been shown to

infect human embryonic kidney cell (Łopata et al. 2018; Lee et al. 2008; Prabha and Verghese 2009), primary human peripheral blood mononuclear cells (Clémenceau et al. 2001; Specke et al. 2001) and primary aortic endothelial cells (Specke et al. 2001) *in vitro*.

A contributing risk factor to transmission of infectious agents by xenografts is the need for immunosuppression of the host's immune system to combat organ rejection. Immunosuppression can result in easier for activation of PERV genomes, replication in transplanted tissue, recombination between PERV variants, possible recombination between PERVs and HERVs, and incorporation of PERVs into the human genome (Bartosch et al. 2004; Patience et al. 1997; Löwer 1999; Wilson et al. 2000). Furthermore, recombination events might support higher levels of integration of recombinant provirus in somatic cells, which can increase the possibility of the infection of pig and human cells by viral particles, and their subsequent spread (Denner and Tönjes 2012). Although rare, such occurrence even at the slightest probability must be taken seriously from a public health perspective.

Understanding of distribution of PERVs in various breeds, in relation to their genomic properties can be helpful in establishing evolutionary history of infections in pigs and their effects. The information gained from this can be applied to making the process of xenotransplantation safer, thus alleviating the shortage of transplant organs. Therefore, study of distribution patterns and evolutionary history is essential to increase the feasibility of pig-human xenotransplantation as a viable medical option. PCR analysis similar to the one used in this study can be further developed to be more sensitive by

designing primers for different sections of the PERV genome, by detecting RT activity, and other molecular detection methods in accordance with the International Xenotransplantation Association recommendations for detection of PERV free animals (Denner et al. 2009).

CONCLUSION

Based on the data obtained from my research, it can be concluded that PERV-C unlike PERV-A and PERV-B is not universal in domestic and feral populations of pigs, with higher percentage of PERV-C positive pigs in the feral population than in domesticated pigs. The data shows that the distribution of PERV-C is not uniform within breeds of domestic pigs. Due to the uneven pattern of distribution of PERV-C in breeds of pigs, it is possible to select individuals from various breeds that are free of PERV-C and use these pigs as source pigs for new herds of PERV-C free pigs. The PERV-C free pigs can be further tested for their PERV-A and PERV-B copy numbers and RNA activity based on which, selection of pigs with low copy numbers of PERV-A and PERV-B can be done. By this selective procedure, pig herds that have lower PERV-A and PERV-B activity and are free of PERV-C can be established having no chances of recombination events between PERV-A and PERV-C which can be used in further research and clinical trials of pig-to-human xenotransplants.

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.

LITERATURE CITED

Bartosch, B., Stefanidis, D., Myers, R., Weiss, R., Patience, C., Takeuchi, Y. 2004. Evidence and Consequence of Porcine Endogenous Retrovirus Recombination. Journal of Virology 78:13880-13890.

Biomedical Research Core Facilities. Sanger Sequencing Information pages URL[https://brcf.medicine.umich.edu/cores/dna-sequencing/faqs/sanger-sequencing-faqs/]. Date accessed [Oct. 2018].

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

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

Clémenceau, B., Jégou, D., Martignat, L., and Saï, P. 2001. Long-term follow-up failed to detect in vitro transmission of full-length porcine endogenous retroviruses from specific pathogen-free pig islets to human cells. Diabetologia 44:2044–2055.

Cozzi, E., Bosio, E., Seveso, M., Rubello, D., 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., 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., 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., 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.

Genewiz. Diagnosis Sanger Interpreting and Troubleshooting Chromatograms. Genewiz, South Plainsfield, New Jersey, USA. Date Accessed: [Oct. 2018].

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

Harrison, I., Takeuchi, Y., Bartosch, B., Stoye, J.P. 2004. Determinants of High Titer in Recombinant Porcine Endogenous Retroviruses. Journal of Virology 78:13871-13879.

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.

Kimsa, M.C., Strzalka-Mrozik, B., Kimsa, M.W., Gola, J., Nicholson, P. Łopata, K., Mazurek, U. 2014. Porcine Endogenous Retroviruses in Xenotransplantation—Molecular Aspects. Viruses 6:2062-2083.

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

Klymiuk, N., Müller, M., Brem, G., Aigner, B. 2002. Characterization of Porcine Endogenous Retrovirus γ pro-pol Nucleotide Sequences. Journal of Virology 76:11738-11743.

Lee, D., Kim, N.Y., Bae, G.E., Lee, H.J., Kwon, M., Kim, S.S., Lee, H.T., Yang, J.M., 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., Gao, Y. 2011. Genetic prevalence of porcine endogenous retrovirus in Chinese experimental miniature pigs. Transplantant Proceedings 43:2762–2769.

Łopata, K., Wojdas, E., Nowak, R., Łopata, P., and Mazurek, U. 2018. Porcine Endogenous Retrovirus (PERV) – Molecular Structure and Replication Strategy in the Context of Retroviral Infection Risk of Human Cells. Frontiers in Microbiology 9:730.

Löwer, R. 1999. The pathogenic potential of endogenous retroviruses: facts and fantasies Trends in Microbiology 7:350-356.

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

Moalic, Y., Blanchard, Y., Félix, H., Jestin, A. 2006. Porcine Endogenous Retrovirus Integration Sites in the Human Genome: Features in Common with Those of Murine Leukemia Virus. Journal of Virology 80:10980-10988.

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., Lee, I.-H. 2017. Inactivation of porcine endogenous retrovirus in pigs using CRISPR-Cas9. Science 357:1303–1307.

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

PHS Guideline on Infectious Disease Issues in Xenotransplantation. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Xenotransplantation/UCM092858.pdf; January 19, 2001.

Prabha, M.S., and Verghese, S. 2009. Polymerase Chain Reaction in Detection of Porcine Endogenous Retrovirus (PERV) from Porcine Tissue. Indian Journal of Microbiology 49:68-71.

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.

Specke, V., Rubant, S., Denner, J. 2001. Productive Infection of Human Primary Cells and Cell Lines with Porcine Endogenous Retroviruses. Virology 285:177-180.

Takeuchi, Y., Patience, C., Magre, S., Weiss, R.A., Banarjee, P.T., Tisser, P.L., 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., Young, .P.R. 2006. Retroviral invasion of the koala genome. Nature 442:79-81

Weiss, R.A. 2006. The discovery of endogenous retroviruses. Retrovirology 3:67.

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

Wilson, C.A., Wong, S., VanBrocklin, M., Federspiel, M.J. 2000. Extended Analysis of the In Vitro Tropism of Porcine Endogenous Retrovirus. Journal of Virology 74:49-56.

Wood, A., Webb, B.L.J., Bartosch, B., Schaller, T., Takeuchi, Y., 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. URL [https://talk.ictvonline.org/ictvreports/ictv_9th_report/reverse-transcribing-dna-and-rna-viruses-2011/w/rt_viruses/162/retroviridae-figures].

TABLES

Sample no	**Crossbreeds**
9-1 to 9-11 23-1 to 23-10	Dark Cross sow 71 by Blue Butt Boar
10-1 to 10-7 22-1 to 22-5	Pure York sow by Blue Butt Boar
19-1 to 19-7	Pure Duroc sow by Berkshire Boar
24-1 to 24-10	Dark Cross (Half Hampshire by Half Duroc) bred by Blue Butt Boar

Table 1. List of sample number and breeds of domestic pigs used for study of PERVs.

Gene	**Primer Sequence**	**Fragment size (bp)**	**Ref**
env-A	F:5′-TGGAAAGATTGGCAACAGCG-3′ R:5′-AGTGATGTTAGGCTCAGTGG-3′	359	Liu et al. (2011)
env-B	F: 5′-TTCTCCTTTGTCAATTCCGG-3′ R:5′-TACTTTATCGGGTCCCACTG-3′	263	Liu et al. (2011)
env-C	F:5′-CTGACCTGGATTAGAACTGG-3′ R:5′-ATGTTAGAGGATGGTCCTGG-3′	281	Liu et al. (2011)

Table 2. Primer sequences used for detection of three distinct variants of the *env* gene PERV-A, PERV-B, and PERV-C from domestic and feral pig tissue samples.

Sample no	Sample size	Results					
		PERV-A		PERV-B		PERV-C	
		Positive	Negative	Positive	Negative	Positive	Negative
9-1 to 9-11 23-1 to 23-10	21	21	0	21	0	6	15
10-1 to 10-7 22-1 to 22-5	12	12	0	12	0	7	5
19-1 to 19-7	7	7	0	7	0	5	2
24-1 to 24-10	10	10	0	10	0	3	7

Table 3. Results of PCR analysis with PERV primer sequences in domestic pig samples obtained from the FHSU farm.

Sample no	Sample size	Results					
		PERV-A		PERV-B		PERV-C	
		Positive	Negative	Positive	Negative	Positive	Negative
P-1 to P-35	35	35	0	35	0	30	5

Table 4. Results of PCR analysis with PERV primer sequences in feral pig samples obtained from Texas.

FIGURES

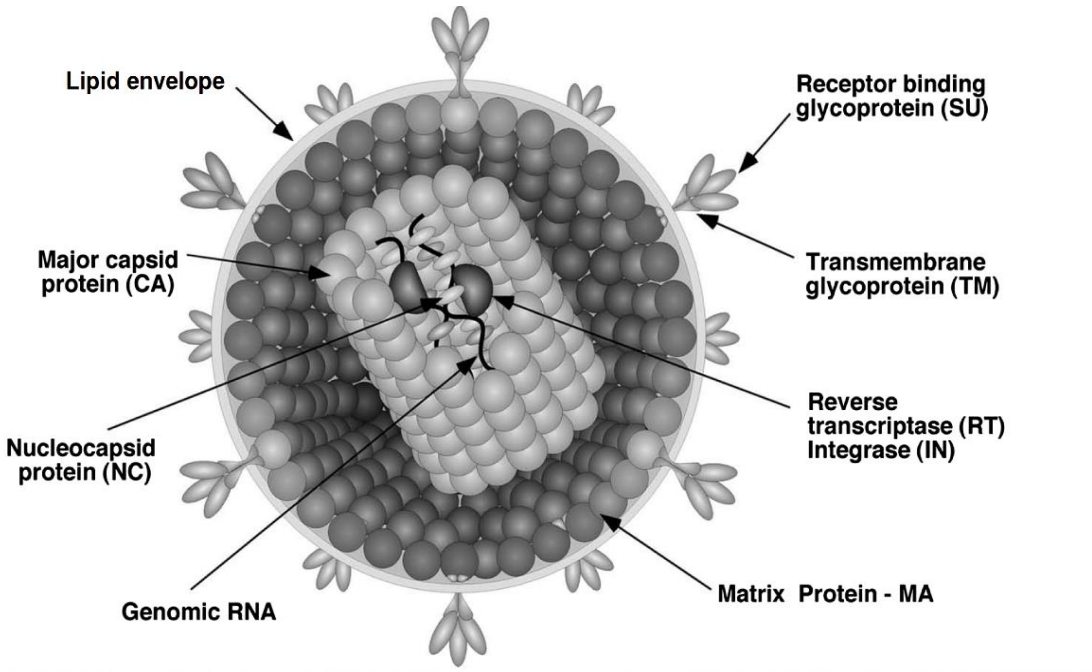

Figure 1. Retrovirus particle (not to scale) illustrating general structure. (Source: Retroviridae-figures, ICTV 9th report, 2011)

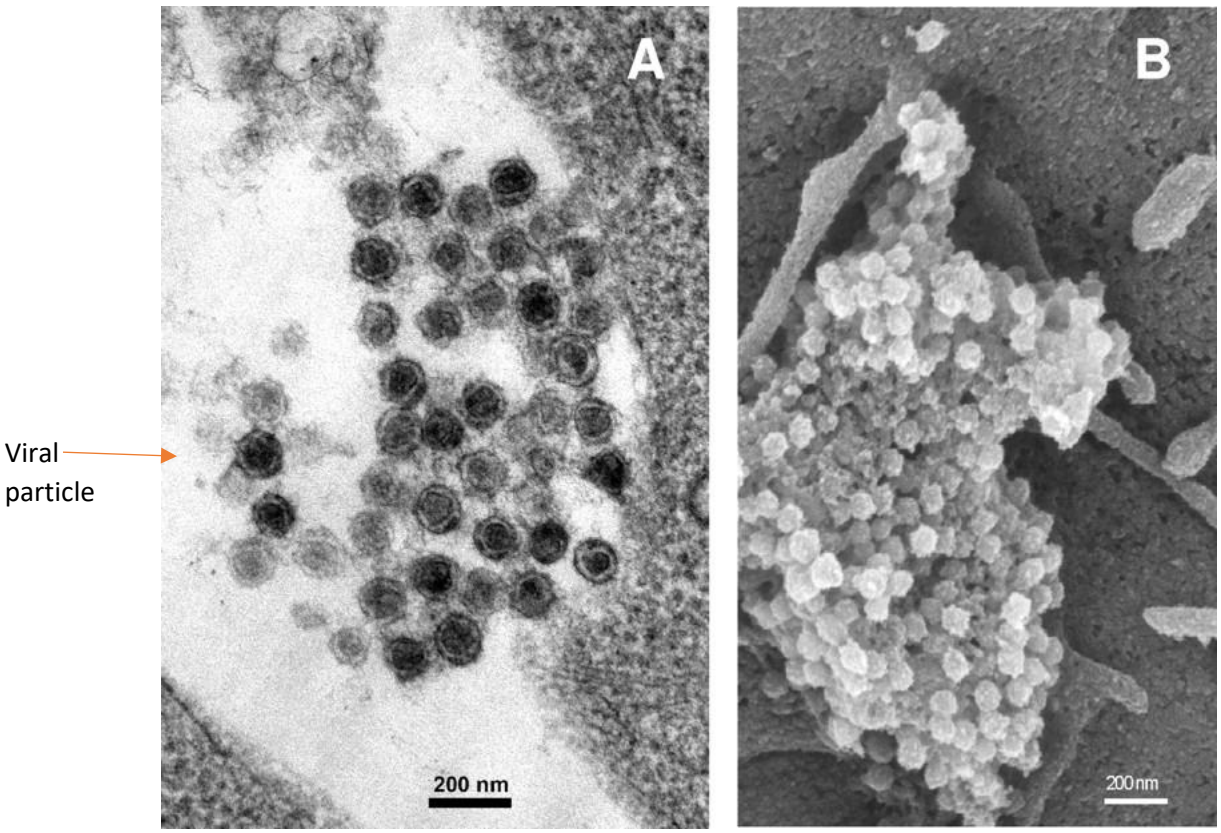

Figure 2. PERVs produced by infected human cells as shown by transmission (A) and scanning (B) electron microscopy. (Source: Denner & Tönjes 2012)

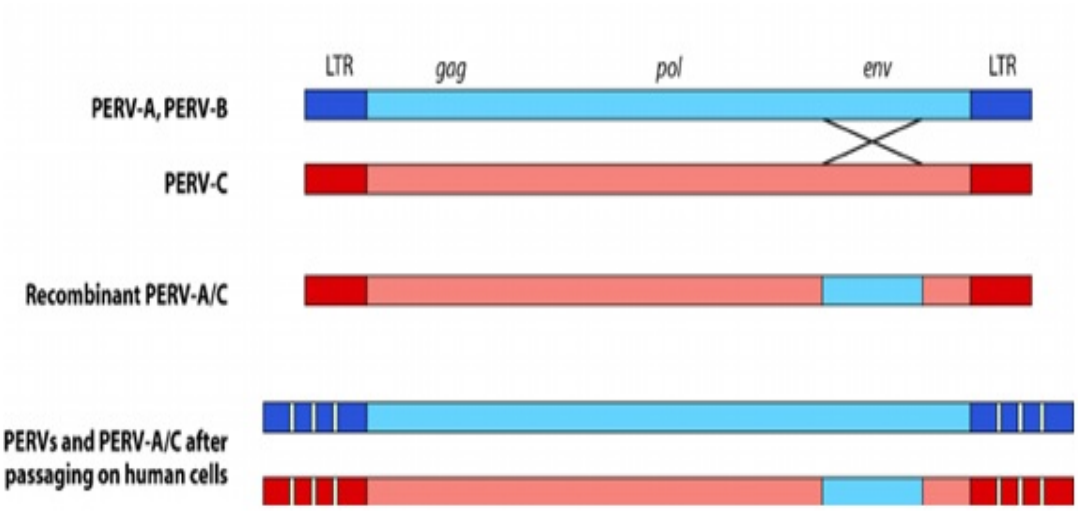

Figure 3. Structure of proviral PERV. Genes and open reading frames are shown as open boxes. LTR, long terminal repeat; *gag*, group-specific antigen gene; ppro/*pol*, protease/polymerase gene; *env*, envelope protein gene. Schematic presentation of the subtypes of PERV and the recombination events and increase in the length of the LTR during passaging on human cells. Boxes in the LTR indicate sequence repeats. (Source: Denner & Tönjes 2012)

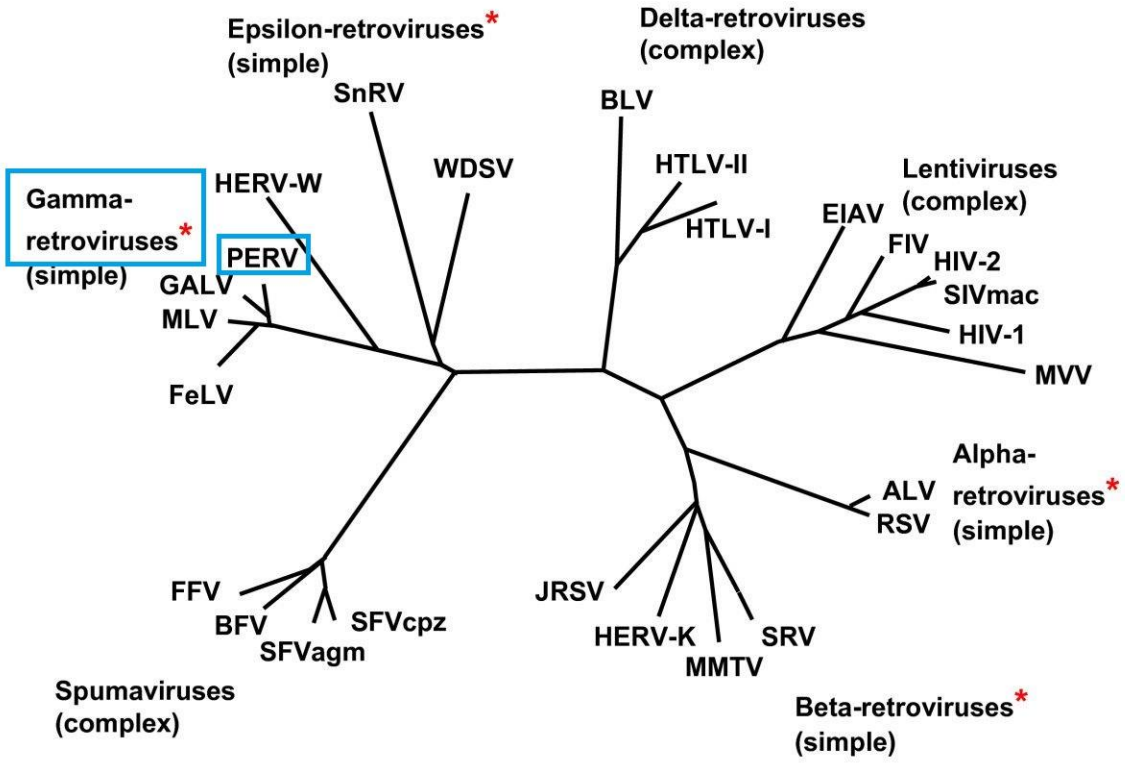

Figure 4. Phylogeny of Retroviruses: genera that include endogenous genomes are marked with an asterisk. Gamma-retroviruses category, and PERV are marked with boxes. (Source: Weiss 2006)

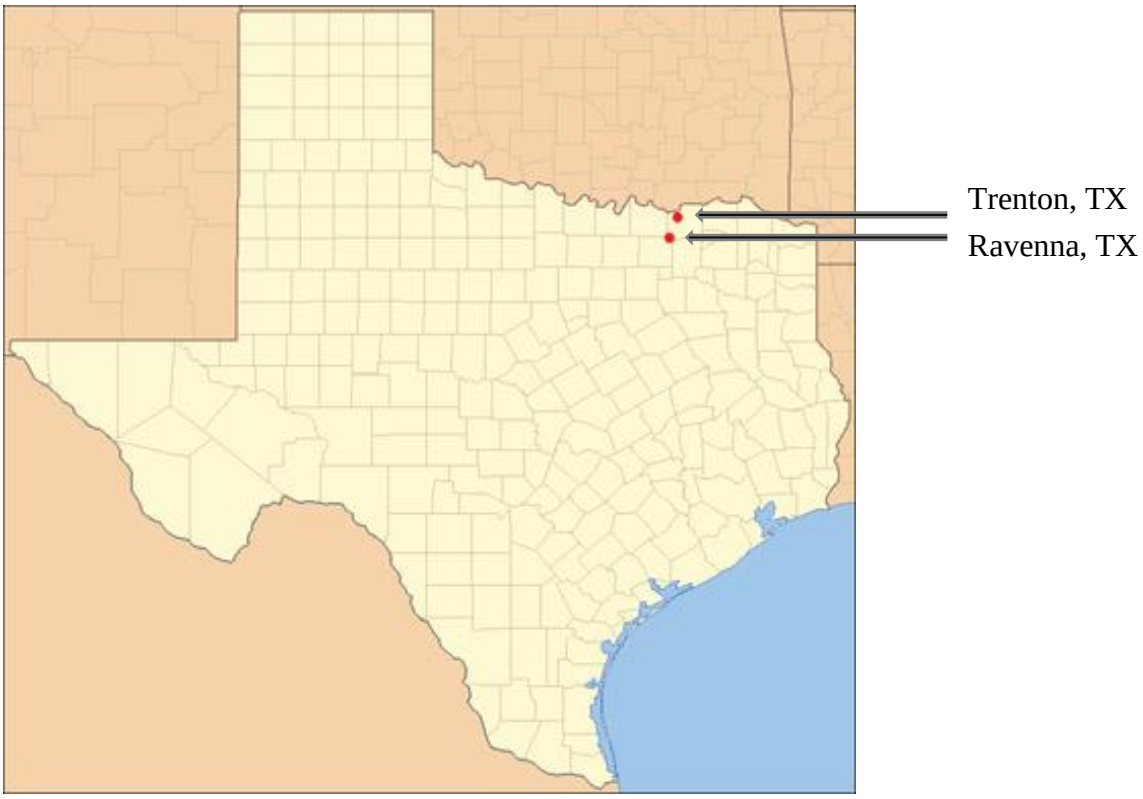

Figure 5. Texas county map showing towns Trenton and Ravenna from where the feral samples were obtained (Source: US Census Bureau)

.

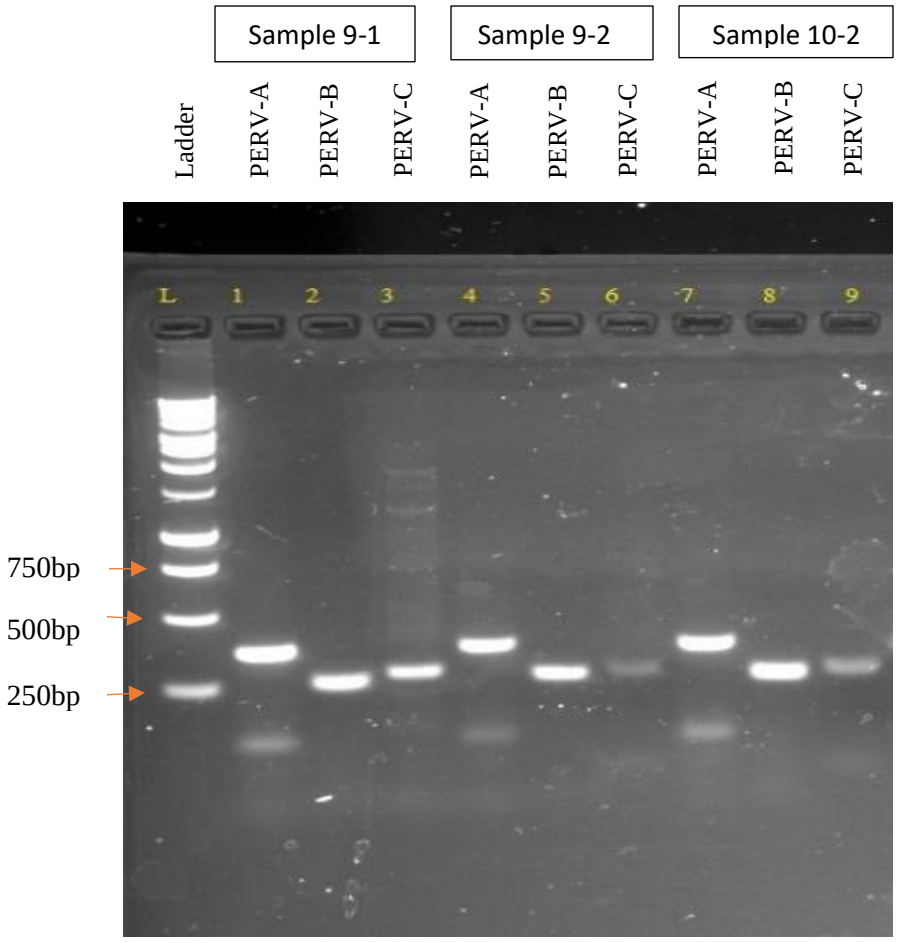

Figure 6. Detection of PERV proviral DNA in domestic porcine tissue samples. Lane L represents the DNA 1 kb DNA ladder. Lanes 1, 2, 3 are for *env*-A, *env*-B and *env*-C respectively for sample 9-1. Lanes 4, 5, 6 are *env*-A, *env*-B and *env*-C for sample 9-2, and lanes 7, 8, 9 are *env*-A, *env*-B, and *env*-C for sample 10-2.

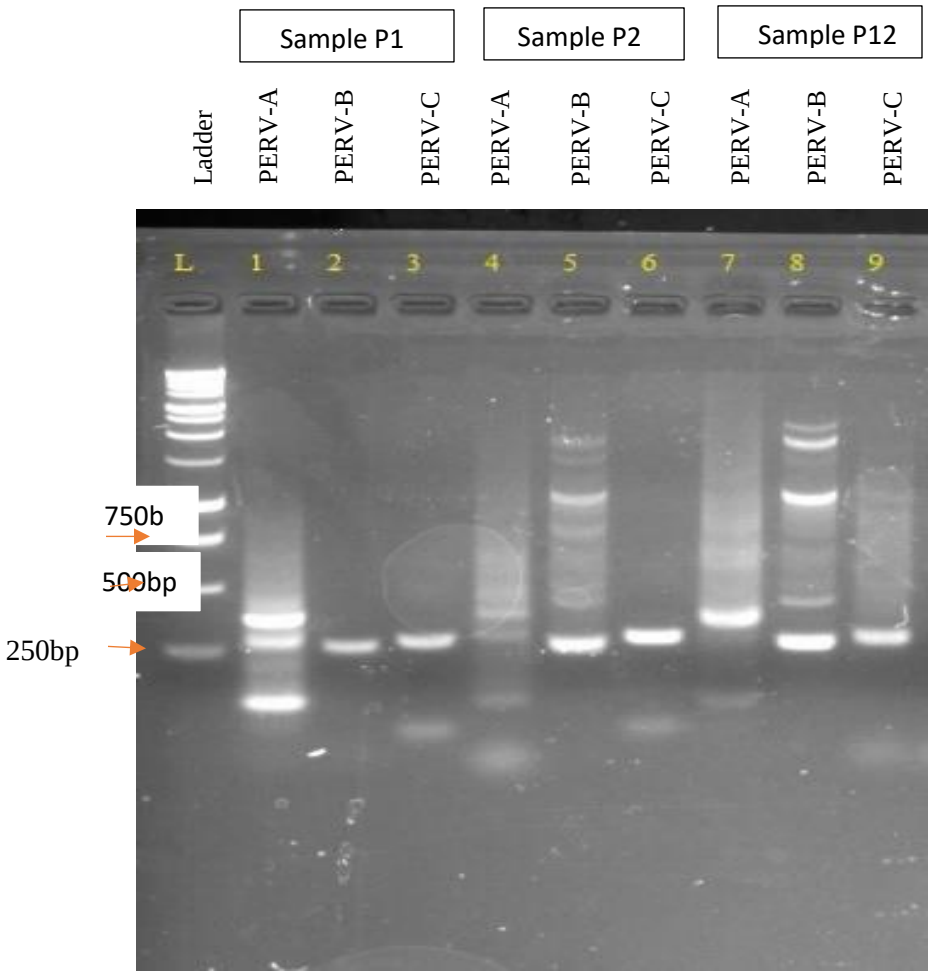

Figure 7. Detection of PERV proviral DNA in feral porcine tissue samples. Lane L represents the 1 kb DNA ladder. Lanes 1, 2, 3 are *env*-A, *env*-B and *env*-C respectively for sample P1. Lanes 4, 5, 6 are *env*-A, *env*-B and *env*-C for sample P2, and lanes 7, 8, 9 are *env*-A, *env*-B, and *env*-C for sample P12.

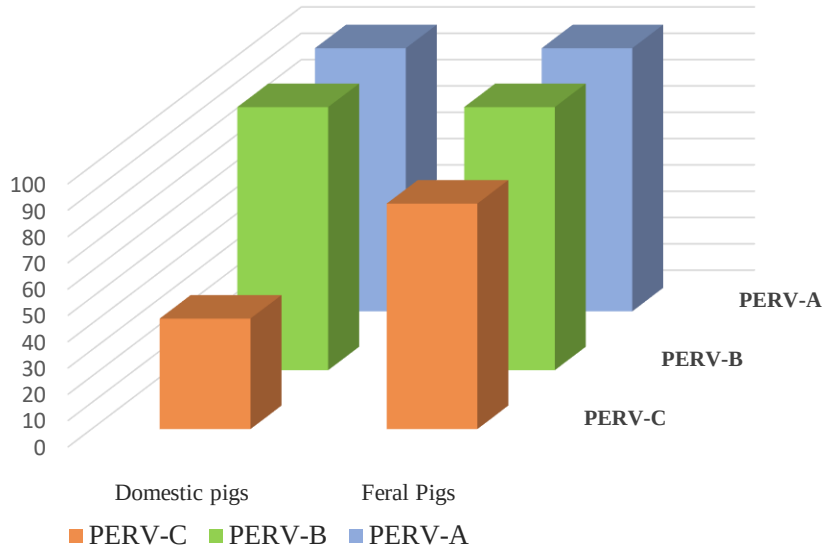

Figure 8. PERV Distribution in domestic and feral pigs in percentage values.

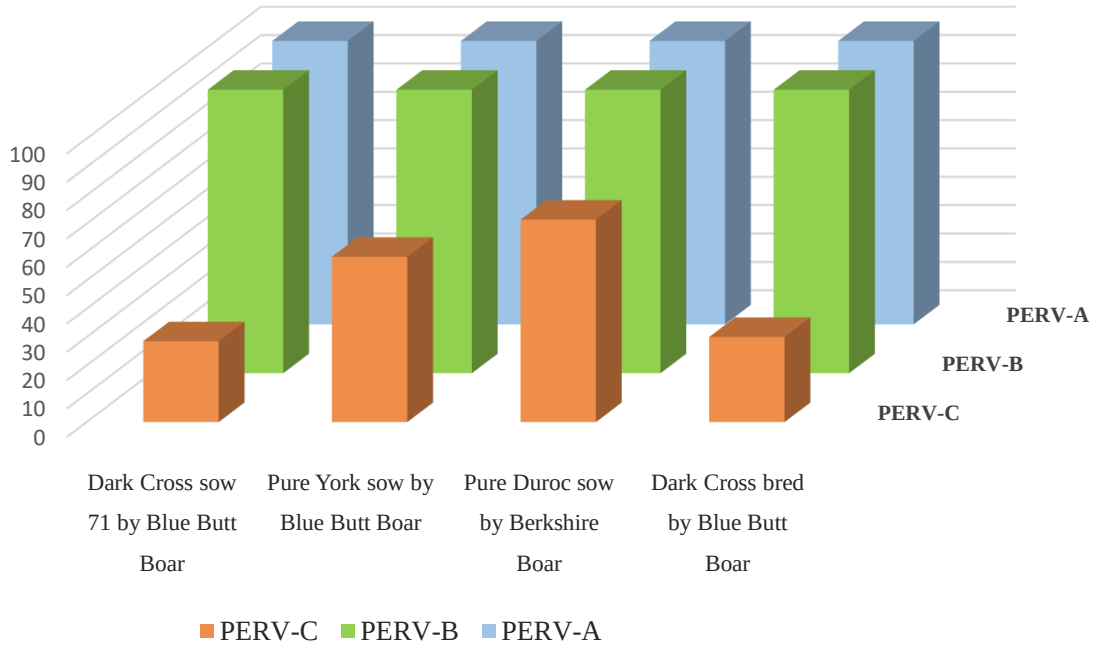

Figure 9. PERV Distribution in domestic breeds of pigs in percentage values.

APPENDIX

Appendix 1. Institutional Care and Use Committee approval.

FORT HAYS STATE UNIVERSITY

Forward thinking. World ready.

Institutional Animal Care and Use Committee

To: Eric Gillock

From Yasuhiro Kobayashi, Institutional Animal Use and Care Committee Chair YK

Re: IACUC protocol (17-0004EX) titled: *Polymorphisims in the PrP prion protein gene in domestic pigs from the FHSU farm*

November 29, 2016

The Institutional Animal Care and Use Committee has reviewed your IACUC protocol application titled: *Polymorphisims in the PrP prion protein gene in domestic pigs from the FHSU farm,* and determined it to be in compliance with all USDA and PHS regulations and requirements and approved.

This approval is for the number and species of animals you listed in the protocol. Your approval will be in effect until November28, 2019.

Please note that the IACUC is required to review and approve, prior to initiation, proposed modifications to an approved protocol.

All approved research protocols must be updated annually, and must be reviewed by the IACUC every three years. All teaching activities using vertebrate animals are reviewed annually.

IACUC approved activities may be subject to further review and approval by university officials; however, those officials may not approve an activity involving the care and use of animals if it has not been approved by the IACUC.

The Principal Investigator is responsible for following federal guidelines and university policies and procedures regarding the care and use of animals.

Please feel free to contact me if there are any questions or concerns regarding the committee's decision on your IACUC protocol.