大韓獸醫學會誌(2009) 第49卷 第3號
Korean J Vet Res(2009) 49(3) : 215~220

Detection of potentially xenozoonotic viruses in the porcine ovary in Korea

Sang-Chul Kang[1], Ji-Youl Jung[1], Hyoung-Seok Yang[1], Bong-Kyun Park[2], Dae-Yong Kim[2], Jae-Hoon Kim[1,*]

[1]*College of Veterinary Medicine and Veterinary Medical Research Institute, Jeju National University, Jeju 690-756, Korea*
[2]*College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea*

(Accepted: August 20, 2009)

Abstract : The prevalence of potentially xenozoonotic viruses in the reproductive tract of female pigs in Korea was investigated by polymerase chain reaction (PCR). These viruses include porcine endogenous retrovirus (PERV), porcine reproductive and respiratory syndrome virus (PRRSV), swine hepatitis E virus (SHEV), porcine lymphotropic herpesvirus (PLHV), and porcine circovirus type 2 (PCV-2). Histopathological examination and PCR analysis were conducted using the ovaries of 70 slaughtered pigs that were collected from 14 farms in Jeju. Histopathologically, infiltrations of mononuclear inflammatory cells around the thick-walled coiled vessels in the ovarian medulla were observed in 15 cases. Based on the PCR method, PERV, PLHV, PRRSV, SHEV, and PCV-2 were detected in 69 (98.6%), 35 (50%), 5 (7.1%), 4 (5.7%), and 1 sample (1.4%), respectively. These results suggest that PERV and PLHV are the major xenozoonotic viruses in the porcine ovary. This study should aid in the development of a monitoring protocol for potential xenozoonotic agents and in the production of germ-free pigs for xenotransplantation.

Keywords : pig ovary, xenotransplantation, xenozoonotic virus

Introduction

Xenotransplantation is the transplantation of living cells, tissues, or organs from nonhuman species to humans. It provides an unlimited and predictable source of organs and allows for immunological pretreatment and the prescreening of organs for various infections [3, 15]. The physiological characteristics of pigs, such as early sexual maturity, a short gestational period, and a large litter size allows for the rapid development of large germ-free breeding herds [3, 15, 17], and recent developments in somatic cell nuclear transfer (SCNT) enable the efficient production of genetically modified cloned pigs for xenotransplantation research. There are, however, a number of problems, including the possible transfer of xenozoonotic pathogens [8], immunological rejection (acute vascular/delayed xenograft and cell-mediated rejection), and ethical concerns [3], that must be overcome before xenobiotic tissues can be used clinically.

Monitoring and preventing xenozoonotic disease is necessary for the successful development of xenotransplantation technologies because animal organs or tissues can transmit pathogens [8]. For SCNT or embryo transfer, it is especially important to be able to identify viruses present in the ovaries and oocytes. Previous studies have identified porcine reproductive and respiratory syndrome virus (PRRSV) and its RNA in ovarian follicles of gilts by immunohistochemistry [19], and porcine circovirus type 2 (PCV-2) DNA has been found in the female reproductive tract and oocytes of PCV-2 antibody-positive pigs [2]. The aim of the present study was to examine porcine ovaries, which are used as donors of oocytes for SCNT, for the presence of potentially xenozoonotic viruses, namely, porcine endogenous retrovirus (PERV), PRRSV, swine hepatitis E virus (SHEV), porcine lymphotropic herpesvirus (PLHV), and PCV-2. Both histopathological

*Corresponding author: Jae-Hoon Kim
College of Veterinary Medicine, Jeju National University, Jeju 690-756, Korea
[Tel: +82-64-754-3387, Fax: +82-64-702-9920, E-mail: kimjhoon@jejunu.ac.kr]

and molecular biological examinations were conducted for efficient detection of viral infection.

Materials and Methods

Ovary samples and histopathology

A total of 70 pig ovaries were selected randomly at a slaughterhouse on Jeju Island and transported immediately to the laboratory. The clinical history of the sows was unknown. Half of each ovary was frozen at −70°C for analysis by polymerase chain reaction (PCR), and the other half was fixed in 10% neutral buffered formalin for histopathology. After fixation, the samples were embedded in paraffin, cut into 3-μm sections, and stained with hematoxylin and eosin.

Extraction of RNA and DNA from samples

Frozen ovaries were analyzed by reverse transcriptase (RT)-PCR for the presence of PERV, PRRSV, and SHEV and by PCR for the presence of PCV-2 and PLHV. The ovaries were washed thoroughly with physiological saline until they were free from blood. Ovary samples (500 mg) were homogenized with 5 mL of DNase- and RNase-free distilled water (Invitrogen, USA), and supernatants were stored at −70°C prior to use. RNA and DNA were extracted from 200 μL of supernatants using an RNeasy Protect Mini Kit (Qiagen GmbH, Germany) and a G-spin DNA extraction kit (iNtRON Biotechnology, Korea), respectively.

RT-PCR and PCR analyses

All PCR analyses were carried out using a Dice TP600 PCR Thermal Cycler (TaKaRa, Japan). For the RT-PCR, 2 μL of RNA samples were added to 18 μL of one-step RT-PCR mixture (Maxime RT-PCR premix; iNtRON Biotechnology, Korea). RT-PCR was carried out as previously described by Czauderna *et al.* [6], Christopher-Hennings *et al.* [5], and Meng *et al.* [16] with minor modifications for the detection of PERV, PRRSV, and SHEV, respectively (Table 1). After one-step RT-PCR, nested PCR for PRRSV and SHEV were performed [5, 16].

For the PCR, 2 μL of DNA samples were added to 18 μL of PCR reaction mixture (Maxime PCR premix; iNtRON Biotechnology, Korea). PCR for PCV-2 and PLHV was carried out as described by Larochelle *et al.* [14] and Ehlers *et al.* [7], respectively (Table 1). The amplified products were visualized by electrophoresis on a 1.2% agarose gel, followed by staining with 0.5 μL/mL ethidium bromide.

Results

Histopathology

Microscopic observation revealed that mild to severe angiocentric nonsuppurative inflammation at the thick-walled coiled vessels in the ovarian medulla were the main histopathologic changes. Among the 70 samples examined, nonsuppurative perivasculitis was observed

Table 1. Oligonucleotide primer sets for the detection of potentially xenozoonotic RNA and DNA viruses

Pathogen	Primer set	Primer sequence (5' to 3')	Amplified product (bp)
PERV	PK1	TTGACTTGGGAGTGGGACGGGTAAC	817
	PK6	GAGGGTCACCTGAGGGTGTTGGAT	
PRRSV	ORF7 F1	TCGTGTTGGGTGGCAGAAAAGC	484
	ORF7 R1	GCCATTCACCACACATTCTTCC	
	ORF7 nF2	CCAGATGCTGGGTAAGATCATC	236
	ORF7 nR2	CAGTGTAACTTATCCTCCCTGA	
SHEV	F1	AGCTCCTGTACCTGATGTTGACTC	404
	R1	CTACAGAGCGCCAGCCTTGATTGC	
	F2	GCTCACGTCATCTGTCGCTGCTGG	266
	R2	GGGCTGAACCAAAATCCTGACATC	
PCV-2	CF8	TAGGTTAGGGCTGTGGCCTT	263
	CR8	CCGCACCTTCGGATATACTG	
PLHV	170S	GCTGACCCAAAGCTCAGGACAATTT	277
	170AS	TATCGCCGTAGATCACCTTGAAGGG	

PERV: porcine endogenous retrovirus, PRRSV: porcine reproductive and respiratory syndrome virus, SHEV: swine hepatitis E virus, PCV-2: porcine circovirus type 2, PLHV: porcine lymphotropic herpesvirus.

in 15 samples. Moderate to severe changes characterized by vascular and perivascular infiltration of lymphocytes and macrophages with fibrinoid necrosis of tunica medina and severe endothelial hypertrophy were occasionally noted (Fig. 1). Follicular atrophy, cessation of mitosis, increased apoptosis in the granulosa cells, separation of the granulosa cell layer from the theca cell layer, and cytoplasmic shrinkage or loss of the oocyte were often observed in the atretic follicles of the ovarian cortex. The antrum was filled with cellular debris, macrophages, and neutrophils.

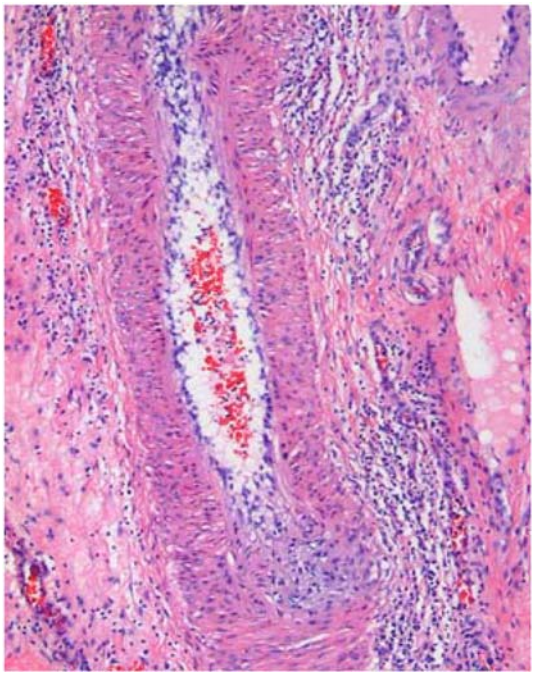

Fig. 1. Moderate infiltration of mononuclear inflammatory cells and endothelial swelling of the thick-walled coiled vessel in the ovarian medulla. H&E, ×100.

RT-PCR and PCR

RT-PCR analyses yielded PCR products corresponding to the expected molecular weights of DNAs from PERV, PRRSV, and SHEV (817, 236, and 266 bp, respectively; Figs. 2 and 3). In the 70 ovaries, the overall positive rates of PERV, PRRSV, and SHEV were 98.6% (69 samples), 7.1% (5 samples), and 5.7% (4 samples), respectively (Table 2). The PCR reactions were expected to produce 263-bp products for PCV-2 and 277-bp products for PLHV (Fig. 4). The positive rates of PCV-2 and PLHV in the 70 ovaries were 1.4% (1 sample) and 50% (35 samples), respectively (Table 2).

Discussion

Our results suggest that potential xenozoonotic viruses, especially PLHV, PERV, and SHEV, were

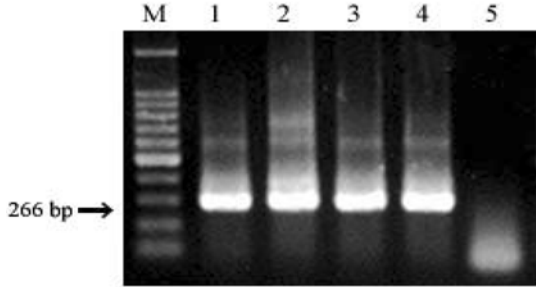

Fig. 3. Nested PCR products of swine hepatitis E virus (SHEV) from ovary and uterus homogenate. Lane M: 100 bp DNA ladder; lane 1-3: 266 bp field samples; lane 4: SHEV positive control; lane 5: SHEV negative control.

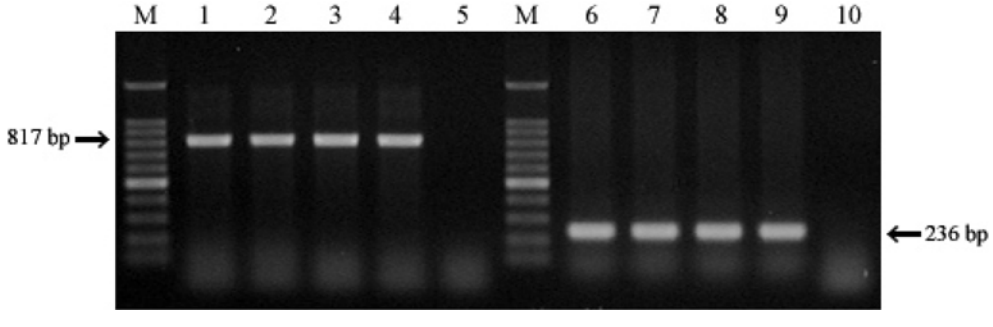

Fig. 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) products of porcine endogenous retrovirus (PERV) and nested PCR products of porcine reproductive and respiratory syndrome virus (PRRSV) from ovary and uterus homogenate. Lane M: 100 bp DNA ladder; lane 1-3: 817 bp field samples; lane 4: PERV positive control; lane 5: PERV negative control; lane 6-8: 236 bp PRRSV field samples; lane 9: PRRSV positive control; lane 10: PRRSV negative control.

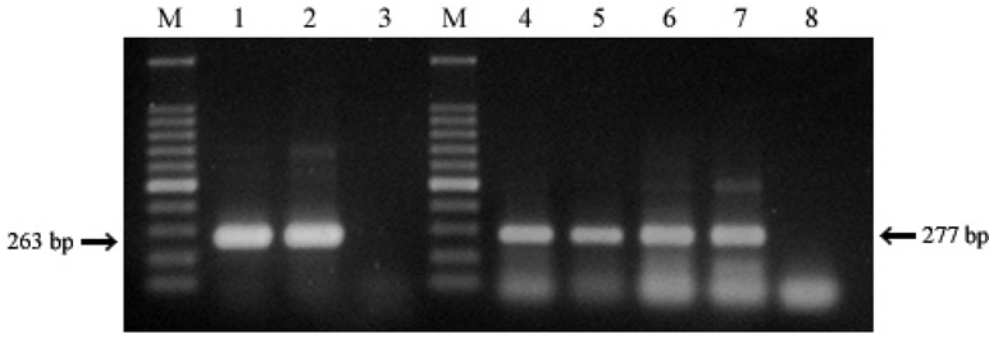

Fig. 4. PCR products of porcine circovirus type 2 (PCV-2) and porcine lymphotropic herpesvirus (PLHV) from ovary and uterus homogenate. Lane M: 100 bp DNA ladder; lane 1: 263 bp field samples; lane 2: PCV-2 positive control; lane 3: PCV-2 negative control; lane 4-6: 277 bp field samples; lane 7: PLHV positive control; lane 8: PLHV negative control.

Table 2. The results of reverse transcriptase-polymerase chain reaction (RT-PCR) for PERV, PRRSV, SHEV and of PCR for PCV-2 and PLHV in the ovaries of 70 slaughtered pigs

Pathogen	PERV	PRRSV	SHEV	PCV-2	PLHV
No. of positive ovaries	69	5	4	1	35
%	98.6	7.1	5.7	1.4	50.0

present in the porcine ovaries. PERV and PLHV were detected in more than half of the samples, whereas less than 8% of the samples were positive for PRRSV, SHEV, and PCV-2. Accordingly, PERV and PLHV appear to be the major xenozoonotic viruses in the porcine ovary.

PERVs are an integral part of pig germ line DNA and can infect and replicate in human cells. Because it is thought that every cell in a porcine xenograft could act as a potential source of a human tropic retrovirus, PERV is a major focus of current xenotransplantation research. These viruses cannot be eliminated by specific pathogen-free breeding or by a simple outcross-breeding protocol. A study of the prevalence of PERV in domestic pigs in Korea found that all of the pigs tested had a high copy number of PERVs within their genomes [13]. We also found a high prevalence of PERV in pig ovaries in the current study.

The current findings are also the first to demonstrate the presence of PLHV in Korea. Sequence analysis has shown that there is high degree of amino acid homology between PLHV-1 and both Epstein-Barr virus and human herpesvirus 8 [7, 20], which are associated with posttransplant lymphoproliferative disorder in human transplant recipients [17]. Furthermore, PLHV-1 genes are transcribed in the lymph nodes of miniature pigs affected by posttransplant lymphoproliferative disorder [10]. Notably, PCR showed that 50% of the ovaries examined in the present study were positive for PLHV DNA. Although newly infected pigs often show no clinical signs of infection, potentially oncogenic PLHV viruses pose another category of risk in xenotransplantation.

A recent study using nested RT-PCR found three types of SHEV and a 2.3% seroprevalence in Korea [4]. Phylogenetic analysis showed that all Korean SHEV isolates were clustered in genotype III, which is similar to that of human and swine HEV isolates from the USA and Japan. Our current results indicated that SHEV is present in the ovary. The US SHEV isolate experimentally infected nonhuman primates, and the US-2 strain of human HEV infected pigs, raising the concern of cross-species infection by SHEV [16]. Based on the zoonotic evidence, SHEV should be eliminated from the tissue before it is used for xenotransplantation.

The genome of PRRSV exists as a linear single-stranded RNA, and the virus is classified as a member of the family *Arteriviridae*, genus *Arterivirus*. There is no evidence that humans can be infected with PRRSV during natural contact with pigs, but it is regarded as a potential pathogen in xenotransplantation [9]. One previous study suggested that PRRSV might contaminate a low proportion of people for up to 48 hours after

direct contact with viremic pigs [1]. They detected PRRSV in 3 people (30%, two saliva samples and one nasal swab) after they were exposed to PRRSV-inoculated pigs. Now PRRSV is not currently defined as zoonotic, it belongs to a family with evidence of frequent changes in host range or pathogenicity. Although the prevalence of PRRSV in pig ovaries was relatively low in this study, the source pig for xenotransplantation would need to be meticulously screened for this virus.

The level of PCV-2 detected in porcine ovary was very low in this study. Infection of human epithelial cells and lymphocytes with PCV-2 causes a cytopathogenic effect, whereas infection with PCV-1 does not cause any visible changes in the cells [11]. According to a previous study, after transfection of adherent human cell lines with DNA of PCV-2, a PCV-specific PCR was performed. And PCV-2 DNA was amplified from several cell lines, such as 293, HeLa, Hep2, RH and Chang liver cells. In addition, a pronounced cytopathic effect was observed in 293 and Hep2 cells but not in PK15 cells [12].

Microscopic observation revealed that 21% of the ovaries showed nonsuppurative inflammation around the vasculature. Viral antigens were detected in all five samples positive for PRRSV by PCR and one sample positive for PCV-2 cases by immunohistochemistry (data not shown), indicating that the inflammatory reactions probably are related to infection with PRRSV or PCV-2.

Viruses that cause symptoms in pigs are relatively easy to eliminate from donor pigs or herds, whereas asymptomatic or latent infections are more difficult to eliminate. Regardless, the ovary should be made free of any viruses that have the possibility to be xenozoonotic. Recently, the Korean Food and Drug Administration listed 32 swine viruses that must be excluded from the organ-source herd for their use in xenotransplantation. When large numbers of ovaries are handled, however, it might not be practical to screen oocytes or embryos for viruses. Therefore, efforts should be centered on careful selection and maintenance of the ovary source herd. Special attention should be paid to possible sources of contamination, including coculture systems or cell culture media.

Acknowledgments

This work was supported by a grant (Code #20070401034009) from BioGreen 21 Program, Rural Development Administration, Korea.

References

1. **Amass SF, Stevenson GW, Anderson C, Grote LA, Dowell C, Vyverberg BD, Kanitz C, Ragland D.** Investigation of people as mechanical vectors for porcine reproductive and respiratory syndrome virus. Swine Health Prod 2000, **8**, 161-166.
2. **Bielanski A, Larochelle R, Algire J, Magar R.** Distribution of PCV-2 DNA in the reproductive tract, oocytes and embryos of PCV-2 antibody-positive pigs. Vet Rec 2004, **155**, 597-598.
3. **Boneva RS, Folks TM, Chapman LE.** Infectious disease issues in xenotransplantation. Clin Microbiol Rev 2001, **14**, 1-14.
4. **Choi IS, Kwon HJ, Shin NR, Yoo HS.** Identification of swine hepatitis E virus (HEV) and prevalence of anti-HEV antibodies in swine and human populations in Korea. J Clin Microbiol 2003, **41**, 3602-3608.
5. **Christopher-Hennings J, Nelson EA, Nelson JK, Hines RJ, Swenson SL, Hill HT, Zimmerman JJ, Katz JB, Yaeger MJ, Chase CCL, Benfield DA.** Detection of porcine reproductive and respiratory syndrome virus in boar semen by PCR. J Clin Microbiol 1995, **33**, 1730-1734.
6. **Czauderna F, Fischer N, Boller K, Kurth R, Tönjes RR.** Establishment and characterization of molecular clones of porcine endogenous retroviruses replicating on human cells. J Virol 2000, **74**, 4028-4038.
7. **Ehlers B, Ulrich S, Goltz M.** Detection of two novel porcine herpesviruses with high similarity to gammaherpesviruses. J Gen Virol 1999, **80**, 971-978.
8. **Fishman JA.** Xenotransplantation from swine: making a list, checking it twice. Xenotransplantation 2000, **7**, 93-95.
9. **Fishman JA, Patience C.** Xenotransplantation: infectious risk revisited. Am J Transplant 2004, **4**, 1383-1390.
10. **Goltz M, Ericsson T, Patience C, Huang CA, Noack S, Sachs DH, Ehlers B.** Sequence analysis of the genome of porcine lymphotropic herpesvirus 1 and gene expression during posttransplant lymphoproliferative disease of pigs. Virology 2002, **294**, 383-393.
11. **Hamel AL, Lin LL, Nayar GPS.** Nucleotide sequence of porcine circovirus associated with postweaning

multisystemic wasting syndrome in pigs. J Virol 1998, **72**, 5262-5267.

12. **Hattermann K, Roedner C, Schmitt C, Finsterbusch T, Steinfeldt T, Mankertz A.** Infection studies on human cell lines with porcine circovirus type 1 and porcine circovirus type 2. Xenotransplantation 2004, **11**, 284-294.
13. **Kim YB, Yoo JY, Lee JY, Kim GW, Park HY.** Prevalence of PERVs from domestic pigs in Korea (pol gene sequences). J Anim Sci Technol 2004, **46**, 307-314.
14. **Larochelle R, Antaya M, Morin M, Magar R.** Typing of porcine circovirus in clinical specimens by multiplex PCR. J Virol Methods 1999, **80**, 69-75.
15. **Magre S, Takeuchi Y, Bartosch B.** Xenotransplantation and pig endogenous retroviruses. Rev Med Virol 2003, **13**, 311-329.
16. **Meng XJ, Halbur PG, Shapiro MS, Govindarajan S, Bruna JD, Mushahwar IK, Purcell RH, Emerson SU.** Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J Virol 1998, **72**, 9714-9721.
17. **Sachs DH.** The pig as a potential xenograft donor. Vet Immunol Immunopathol 1994, **43**, 185-191.
18. **Setsuda J, Teruya-Feldstein J, Harris NL, Ferry JA, Sorbara L, Gupta G, Jaffe ES, Tosato G.** Interleukin-18, interferon-γ, IP-10, and Mig expression in Epstein-Barr virus-induced infectious mononucleosis and posttransplant lymphoproliferative disease. Am J Pathol 1999, **155**, 257-265.
19. **Sur JH, Doster AR, Galeota JA, Osorio FA.** Evidence for the localization of porcine reproductive and respiratory syndrome virus (PRRSV) antigen and RNA in ovarian follicles in gilts. Vet Pathol 2001, **38**, 58-66.
20. **Ulrich S, Goltz M, Ehlers B.** Characterization of the DNA polymerase loci of the novel porcine lymphotropic herpesviruses 1 and 2 in domestic and feral pigs. J Gen Virol 1999, **80**, 3199-3205.