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ORIGINAL ARTICLE

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Extended microbiological characterization of Göttingen minipigs: porcine cytomegalovirus and other viruses

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Abstract

Background: To prevent transmission of zoonotic microorganisms from pig transplants to human recipients when performing xenotransplantation using pig cells, tissues, or organs, donor pigs have to be carefully characterized. Göttingen minipigs (GöMP) are often used for various biomedical investigations and are well characterized concerning the presence of numerous bacteria, fungi, viruses, and parasites. Recently, we studied the prevalence and expression of porcine endogenous retroviruses and the prevalence of hepatitis E virus (HEV) in GöMP. Here, we studied the presence of the porcine cytomegalovirus (PCMV) and porcine lymphotropic herpesviruses (PLHV) and extended testing for hepatitis E virus (HEV).

Methods: PCR, nested PCR, real-time PCR, real-time RT-PCR, and Western blot analyses were used to estimate the prevalence of PCMV, PLHV-1, PLHV-2, PLHV-3, and HEV.

Results: Using different PCR methods, and different source materials, PCMV was found in 10 of 26 adult GöMP, which had been derived originally by cesarean section and kept under specified pathogen-free conditions. Only highly sensitive methods gave positive results, not methods of lower sensitivity. The virus load in all positive animals was low (<100–200 copies per mL). PLHV-1, PLHV-2, and PLHV-3 were not detected by PCR; however, an anti-PLHV immune response was found in one of 10 animals tested by Western blot analyses. HEV was detected by RT-PCR in two of nine tested animals, but no anti-HEV immune response was observed.

Conclusion: Using highly sensitive methods, PCMV, HEV, and PLHV were found in some GöMP, suggesting that these viruses may be introduced through the placenta. The results show that highly sensitive methods are required to characterize pigs to be used for xenotransplantation to prevent virus transmission.

KEYWORDS

hepatitis E virus, microorganisms, porcine cytomegalovirus, porcine herpesviruses, screening, viral safety

1 | INTRODUCTION

Xenotransplantation may help to overcome the shortage of human transplants for the treatment of organ failure. For several reasons, pigs are considered as donor animals. In the last years, a significant progress was achieved in generation of multitransgenic animals to prevent immunological rejection of the transplant (for review, see Ref.[1–3]). However, xenotransplantation using pig cells, tissues, and organs may be associated with the transmission of potentially zoonotic porcine microorganisms to the recipient.[4,5] In addition to the

prevention of rejection and the prevention of transmission of zoonotic microorganism, xenotransplantation has to overcome another hurdle, physiologic incompatibility of the organs. As organs from conventional pigs may be too large, those from minipigs may be better suited. At the moment, the Göttingen minipigs (GöMP) are well characterized from the microbiological point of view.[6–8] GöMP are the result of crossbreeding the Minnesota minipig, the Vietnamese potbelly pig, and the German Landrace pig. This breed is used in biomedical research and may be considered as donor of islet cells and organs. The herd bred at Ellegaard (Denmark) is produced in a full-barrier specified pathogen-free (spf) facility, and physiologic parameters and health status of the animals are well defined.[6,7] GöMP are screened twice a year for numerous microorganisms including 27 bacteria, 16 viruses, three fungi, and four parasites (http//www.minipigs.dk/). We recently characterized the prevalence and expression of porcine endogenous retroviruses (PERVs) in GöMP.[8] Whereas nearly all of the other microorganisms may be eliminated by spf or designated pathogen-free (dpf) breeding of the animals, PERVs cannot be eliminated because they are integrated in the genome of all pigs (for review, see Ref.[9,10]). Furthermore, we studied the prevalence of 88 other microorganisms and found that hepatitis E virus (HEV) was present in a small number of GöMP.[11] Whereas HEV was not detected in adult animals and retired breeders, it was found in some younger GöMP, in sows after giving birth, and in their offspring, indicating a sow-to-piglet HEV transmission.[11] The pathogenic potential of porcine cytomegalovirus (PCMV) and porcine lymphotropic herpesviruses (PLHV-1, PLHV-2, PLHV-3) for humans is unclear, but it is well known that herpesviruses may be latent in adult animals and the virus titer is frequently very low (for review, see Ref.[12,13]). In this regard, highly sensitive diagnostic methods are essential to detect these viruses. Here, results of the screening of GöMP for additional microorganisms, including PCMV and PLHV, using newly established highly sensitive methods are presented.

2 | MATERIALS AND METHODS

2.1 | Animals and tissue samples

Liver and kidneys from five minipigs and whole blood and serum from 21 Göttingen minipigs were obtained from the SPF facility (Ellegaard, Denmark).

2.2 | Extraction of DNA

DNA was extracted from organs using DNeasy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany) and from sera or blood using the DNA extraction kit mentioned above and the ZR viral DNA kit (Zymo Research Corp., Irvine, CA, USA). For testing, DNA extraction from sera and blood was performed three times, using each time 100 µL. The DNA was quantified on NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific, Inc. Worcester, MA, USA).

2.3 | PCR, nested PCR, and real-time PCR for testing PCMV

DNA extracted from kidneys and livers was tested for PCMV using (i) a conventional PCR developed by Goltz et al.,[14] (ii) a newly established nested PCR, and (iii) a modification of a previously established real-time PCR [15] (for primers, see Table 1). For the nested PCR, the GoTaq Green master mix (Promega, Madison, WI, USA) was used according to the manufacturer's instructions. A total of 300 ng of each DNA sample from the organs was tested in reaction mixture with 10 µmol/L of PCMV-specific primers (PCMV F1 EP and PCMV R1 EP, Table 1) in a total volume of 25 µL. The thermal cycling conditions were the following: polymerase activation for 2 minutes at 95°C and 35 cycles of amplification with denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds and elongation at 72°C for 1 minute. The final elongation step was carried out for 7 minutes. One microliter from the first PCR was used in nested PCR. Conditions of the second round with primers PCMV F2 EP and PCMV R2 EP (Table 1) were similar, but annealing temperature was reduced to 56°C. The previously established real-time PCR[15] was modified as a duplex real-time PCR with changed parameters as described below. A total of 100 ng of each DNA was used. The PCR mixture contained the TaqMan Universal PCR 2× master mix (Life Technologies, Carlsbad, CA, USA), 900 nmol/L of the PCMV-specific primers, 450 nmol/L of pGAPDH-specific primers, and 200 nmol/L of each probe in a total volume of 25 µL. The conditions were as follows: enzyme activation for 10 minutes at 95°C, followed by 45 cycles of denaturation at 95°C for 20 seconds and annealing/extension at 59°C for 1 minute. Reaction was performed in a Stratagene MX3005P qPCR system (Agilent Technologies, Santa Clara, CA, USA).

A total of 100 ng of DNA from sera and blood was tested using recently established highly sensitive nested PCR and real-time PCR systems (system 2) with estimated detection limits of 5 and 2 copies, respectively.[16] A standard curve was generated as described,[16] using cloned PCR amplified PCMV fragment. Reporter fluorescence was measured using a Stratagene Mx3005P qPCR system (Agilent Technologies).

2.4 | PCR and real-time PCR for testing PLHV

PLHV PCRs were performed using the primer 747s and 747as for the detection of PLHV-1 and PLHV-2 (B. Ehlers, personal communication), and using the primers 905s and 905as for the amplification of PLHV-3[17] (Table 1). The AmpliTaq Gold® polymerase was activated for 12 minutes at 95°C followed by 45 PCR cycles: denaturation for 30 seconds at 95°C, annealing for 30 seconds at 56°C (primers 747s/747as, PLHV-1, PLHV-2), or 57°C (primers 905s/905as, PLHV-3), and extension of 1 minute or 2 minutes at 72°C depending on amplicon size. The final extension step was carried for 15 min. Amplicons were examined on 1.2% agarose gel, and the GeneRuler™ 100-bp DNA ladder (Thermo Scientific, Schwerte, Germany) was used for gel calibration.

TABLE 1 Primers and probes used for PCR

Primer used for PCR	Sequence 5′–3′	Reference	Nucleotide position (GenBank acc. number)
PLHV-1,-2 747 S PLHV-1,-2 747 AS	CAYGGTAGTATTTATTCAGACA GATATCCTGGTACATTGGAAAG	Ehlers et al. (personal communication)	21146-21167 (AY170317.1) 21488-21467
PLHV-3 905-s PLHV-3 905-as	ACAAGAGCCTTAGGGTTCCAAACT GTGTCCAGTGTTGTAATGGATGCC	Chmielewicz et al.,[17]	13472-13495 (AY170316.1) 13727-13704
PCMV 199 PCMV 199AS	TCTAGACGAAAGGACATTGTTGATA ACGAGAAAGATATTCTGACGGTGCA	Goltz et al.,[14]	45622-45646 (KF017583.1) 45962-45938
PCMVDNA pol F1 PCMVDNA pol R1	ACGGGGATCGACGAGAAAG CTAGACGAGAGGACATTGTTGAT	Morozov et al.,[16]	63-81 (AJ222640) 412-390
PCMVDNA pol F2 PCMVDNA pol R2	GAAGAGAAAGGAAGTGAAGG GTCACTCGTCTGCCTAAGC	Morozov et al.,[16]	182-201 (AJ222640) 386-368
PCMV F1 EP PCMV R1 EP	GTCAAGAACATCGTGCCCGAGA ACAGCATGGTGGACAGGACAA	This manuscript	45522-45501 (KF017583.1) 45087-45107
PCMV F2 EP PCMV R2 EP	AGCTCTCTCAGATGAGCTGCG CCTATCCCTCGCGCCAATTA	This manuscript	45233-45213 (KF017583.1) 45139-45158
Primers and probes used for real-time PCR			
pGAPDH fw pGAPDH rev pGAPDH probe	ACATGGCCTCCAAGGAGTAAGA GATCGAGTTGGGGCTGTGACT7 HEX-CCACCAACCCCAGCAAGAG-BHQ1	Duvigneau et al.,[29]	1040-1062 (NM001206359.1) 1188-1168 1114-1132
PCMV real fw PCMV real rev PCMV probe	ACTTCGTCGCAGCTCATCTGA GTTCTGGGATTCCGAGGTTG 6FAM-CAGGGCGGCGGTCGAGCTC-TAMRA	Mueller et al.,[15]	45206-45226 (KF017583.1) 45268-45249 45246-45229
PCMV DNApol Fr-t PCMV DNApol Rr-t PCMV DNApol probe	AATGCGTTTTACAACTTCACG CTGAGCATGTCCCGCCCTAT 6FAM-CTCTAGCGGCGTCCATCACC-BHQ/2	Morozov et al.,[16]	279-299 (AJ222640) 373-354 331-350
JVHEVF JVHEVF JVHEVP	5′-GGTGGTTTCTGGGGTGAC 5′-AGGGTTGGTTGGATGAA 6-FAM-TGATTCTCAGCCCTTCGC-BHQ	Jothikumar et al.[18]	5261-5278 (M73218) 5330-5313 5284-5301

2.5 | Cloning and sequencing

PCR amplicons were ligated into the pCR 2.1-TOPO vector according to the protocol of the supplier (Invitrogen Life Technologies, Carlsbad, CA, USA). Z cells (Zymo Research Corp.) were transformed with the constructs and plated on LB agar/ampicillin dishes for 18 hours at 37°C. Five clones from each dish were collected and amplified in LB/ampicillin medium overnight at 37°C. Plasmids were isolated using PureYield Plasmid Miniprep System (Promega) and sequenced in both directions using primers from the cloning kit and BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA).

2.6 | PCMV testing at Zoologix

In parallel, sera and blood samples from adult mini pigs were tested for PCMV at Zoologix (Chatsworth, CA, USA), using corresponding real-time PCR approaches (http://www.zoologix.com/).

2.7 | Reverse transcriptase (RT) real-time PCR for detection of hepatitis E virus

HEV detection was performed as described.[18] RNA was extracted from 100 µL of sera using RNeasy mini kit (Qiagen, Hilden, Germany) and from 200 µL of sera using ZR viral RNA kit (Zymo Research Corp.), respectively.

2.8 | Recombinant proteins used as antigen for the Western blot analysis

A recombinant protein corresponding to the N-terminal part of the PLHV glycoprotein B1 was used to test for anti-PLHV IgG immune response. The protein was produced as His-tagged fusion protein in *E. coli* Rosetta (DE3) pLacI cells transformed with P1-pTriEXgB1 as described.[19] Protein was purified and characterized by SDS-PAGE. The protein was used to immunize a goat to obtain a positive serum.[20] For the detection of anti-HEV IgG by Western blot analysis, two recombinant proteins (GT3 and Prospec) corresponding to ORF 2 of HEV were used together as described.[11]

2.9 | SDS-PAGE and Western blot analyses

In brief, SDS-PAGE was performed in precast preparative Tris–glycine 4%–20% gradient SERVAGels (Serva Electrophoresis GmbH, Heidelberg, Germany) as described. The load of antigens per gel was calculated to obtain finally 300 ng per membrane strip. After electrophoresis and transfer, the membranes we cut in strips and were

TABLE 2 Analysis of liver (L) and kidney (K) of GöMP for PCMV and PLHVs using PCR, nested PCR, and real-time PCR

						PCMV		PLHV[a]		
Number	Barrier	Age (years)	Sex	Material[b]	PCR[c]	Nested PCR system 1	Real-time PCR[d]	PLHV-1	PLHV-2	PLHV-3
313266	3	1	F	L/K	+/+	+/+	+ (Ct 32)/+ (Ct 35)	−/−	−/−	−/−
217288	2	1	M	L/K	−/−	−/−	−/−	−/−	−/−	−/−
217271	2	1	M	L/K	−/−	−/−	−/−	−/−	−/−	−/−
217275	2	1	M	L/K	−/−	−/−	−/−	−/−	−/−	−/−
217200	2	1	F	L/K	−/−	−/−	−/−	−/−	−/−	−/−

[a]PCR assays that allow to detect PLHV-1,2 (Ehlers et al., personal communication) or PLHV3.[17]

[b]DNA was isolated from each organ three times and tested independently.

[c]Goltz et al.[14]

[d]Mueller et al.,[15] performed as a duplex PCR. The Ct values of the positive samples were given, "no Ct" in the case of all negative samples.

blocked with 6% dry milk in PBS with 0.1% Tween 20 (blocking buffer) overnight at 4°C. Strips were incubated with sera diluted 1:150 in blocking buffer for 2 hours at room temperature. Goat anti-pig IgG (Abcam, Cambridge, UK) alkaline phosphatase conjugated antibodies (Sigma-Aldrich, St. Louis, MI, USA) were taken 1:1000 in blocking buffer. Reaction was developed using NBT (nitro-blue tetrazolium chloride)—BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) substrate (Promega). Sera from a HEV-infected and a non-infected pig were used as positive and negative controls, respectively.

3 | RESULTS

3.1 | Testing for PCMV

The prevalence of PCMV in GöMP was assessed by different PCR approaches using DNA extracted from liver, kidney, whole blood, and sera. DNA from kidney and liver from five GöMP were examined using a (i) conventional PCR described previously,[14] (ii) a newly established nested PCR (system 1), and (iii) a real-time PCR.[15] The DNA was isolated and tested three times independently, and only one animal (313266) was found positive in these assays (Table 2).

As for the screening of animals in breeding programs only blood and sera are available, a sensitive nested PCR and a real-time PCR (system 2) with a sensitivity about 5 and 2–5 copies PCMV/reaction, respectively, were developed [16] and used for testing. DNA was isolated from sera of 10 GöMP of different ages and was tested using real-time PCR system 1. Only one animal was found positive. However, when the same DNA was tested using real-time PCR system 2, five animals (two males and three females) were found positive for PCMV (Table 3). The Ct values were between 34.5 and 37.6, indicating that the virus load was approximately 5 to 6 copies and to 2 copies of PCMV genome equivalents per reaction, respectively. Counting the amount of sera taken for DNA extraction and the amount of DNA used for a reaction, it appears that <200 PCMV genome equivalents were present in one ml of sera. Our results demonstrate that PCMV-positive animals were found in both barriers of the spf facility (Table 3) (the first figure in the animal number indicates the barrier 2 or 3). In a next experiment, 11 adult animals, among them retired breeders, were tested also using DNA from sera. The tests were performed using two methods, nested PCR system 2 and real-time PCR system 2 (Table 4). Three animals were found positive using nested PCR (Fig. 1), and four were found positive using the real-time PCR (Table 4). It should be emphasized that three of four PCR-positive animals were detected in both tests. The specificity of the PCR amplicons from animals 222031, 318208, and 314253 was confirmed by sequencing of the amplicons and clones. All PCMV sequences were identical and differed by one nucleotide from the sequence used as a reference (GenBank acc. #AJ222640). In parallel, the same samples were tested by PCR at Zoologix. None of the animals were shown positive.

3.2 | Testing for PLHV

To analyze the prevalence of PLHV-1, PLHV-2 and PLHV-3, a conventional PCR was used[17] (B. Ehlers, personal communication). The selected primers allow testing for all three PLHV and to discriminate between PLHV-1 and PLHV-2 on one hand and PLHV-3 on the other (Fig. 2). Previously, DNA from sera of 10 GöMP was tested for the presence of PLHV and found to be negative.[20] In addition, DNA from liver and kidney of five other animals was tested here and found negative (Table 2). Furthermore, sera from 10 new animals were tested and found also negative (Table 3). Next, a Western blot analysis was performed using a recombinant protein representing a part of the gB1 glycoprotein that is recognized by sera against all three PLHV.[21] Sera from 10 animals were tested, and serum from animal 320002 was found positive (Fig. 2), indicating that at least one animal could be PLHV infected.

3.3 | Testing for HEV

Nine adult animals were tested by RT real-time PCR for HEV infection using RNA from sera. Parameters of reaction and primers used

TABLE 3 PCMV and PLHV markers in the blood and serum from GöMP of different ages as detected by PCR, real-time PCR, and Western blot analysis

Animal[a]	Barrier	Gender	Age (months)	PCMV Real-time PCR (blood) system 1	PCMV Real-time PCR[b] (sera) system 2	PLHV PCR[c] (sera)	PLHV Western blot
221907	2	M	5	–	–	–	–
220958	2	M	10	–	–	–	–
220348	2	M	11	–	+ (Ct 36)	–	–
320002	3	M	5	–	+ (Ct 37)	–	+
318341	3	M	10	–	–	–	–
222196	2	F	3	–	–	–	–
221368	2	F	7	–	+ (Ct 34)	–	–
319395	3	F	7	–	+ (Ct 35)	–	–
319332	3	F	7	–	–	–	–
317839	3	F	12	+ (Ct38)	+ (Ct 37)	–	–
Total				1/10	5/10		

[a]The first figure of the animal number indicates barrier 2 or 3.
[b]The Ct values of the positive samples were given, no Ct values for all negative samples.
[c]PCR assays that allow to detect PLHV-1,2 (B. Ehlers et al., personal communication) or PLHV3.[17]

TABLE 4 PCMV and HEV markers in the sera of GöMP as detected by nested and real-time PCR as well as Western blot analysis

Animal	Barrier	Sex	Age (months)	PCMV Nested PCR system 2	PCMV Real-time PCR system 2[a]	PCMV Real-time PCR Zoologix	HEV Real-time RT-PCR [a,b]	HEV Western blot
320203	3	F	10	–	–	–	+ (Ct 38)	–
220407	2	F	17	–	–	–	–	–
318208	3	F	16	+	+ (Ct 38)	–	+ (Ct 40)	–
222031	2	M	10	+	+ (Ct 36)	–	–	–
319345	3	M	13	–	–	–	–	–
216646	2	F	32	–	+ (Ct 40)	–	–	–
219748	2	M	18	–	–	–	–	–
312971	3	F	30	–	–	–	–	–
221806	2	F	11	–	–	–	–	–
314253	3	F	20	+	+ (Ct 33)	–	*	*
314451	3	F	18	nt	–	–	*	*
Total				3/10	4/11		2/9	0/9

*HEV-positive as tested previously[11]; nt, not tested.
[a]The Ct values of the positive samples were given, "no Ct" values in the case of all negative samples.
[b]Jothikumar et al.[18]

were as described[18] (Table 4). Two animals were found HEV positive, but the virus load was very low. The Ct values corresponded to approximately 5–10 HEV genome equivalents in the reaction, or less than 100 copies per ml. In addition, two animals previously tested positive[11] were retested and found again positive (Table 4). Western blot analysis using pig sera revealed no IgG immune response against two recombinant ORF2 proteins of HEV (data not shown).

4 | DISCUSSION

Using highly sensitive methods, about one-third of the GöMP tested were found infected with PCMV and antibodies against PLHV were found in at least one animal. Whereas the PCR methods used were able to discriminate between PLHV-1, PLHV-2 or PLHV-3 (Fig. 2), the Western blot analysis using a cross-reacting antigen was not, and therefore, it remains unclear which PLHV infected the positive animal

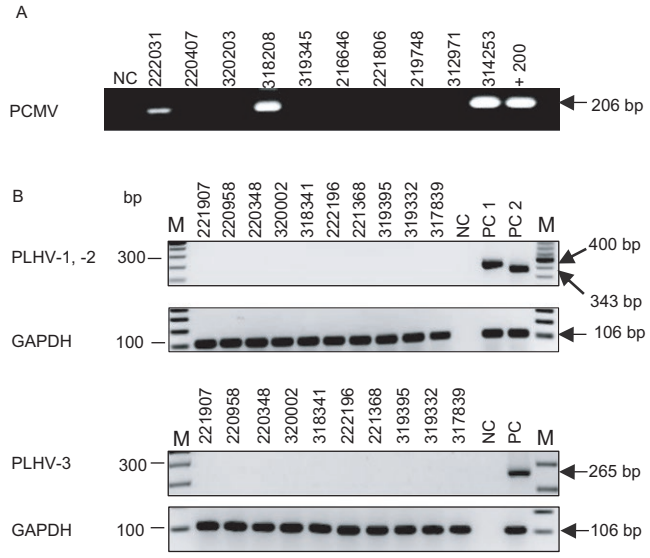

FIGURE 1 (A) Detection of PCMV infection by nested PCR (system 2)[16] in a group of adult animals. NC, negative control (water), +200—positive control that contained 200 copies of the reference plasmid. Amplicons from the positive samples 222 031, 318 208 and 314 253 were cloned and sequenced. (B) Conventional PCR screening for PLHV infection. NC, negative control, PC1, positive control for PLHV-1, PC2, positive control for PLHV-2, PC, positive control for PLHV-3, M, 100-bp DNA ladder

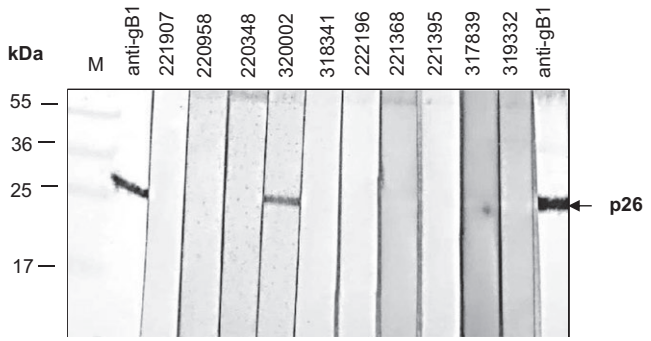

FIGURE 2 Western blot analyses of pig sera using as antigen the recombinant protein p26 corresponding to a part of the gB1 glycoprotein of the PLHV-1. Ten sera from GöMP of different ages (Table 3) were tested at dilution 1:100. A positive control serum was produced in a goat immunized with recombinant p26 gB1 and was used at a dilution 1:1000. Sera from the adult animals including retired breeders (Table 4) were anti-p26 gB1 negative (not shown). Position of the target protein is indicated by an arrowhead. M—size markers in kilodaltons (kDa)

and induced the immune response. Although antibodies against PLHVs were detected only in one animal, the strong reactivity argues against unspecific cross-reactivity.

PCMV is frequent, but mostly latent in adult pigs. This latency poses a significant difficulty in virus detection, especially if only blood or sera are available. Thus, to meet this challenge, highly sensitive PCR detection systems were established and used in this study.

The risk of infection of humans with PCMV was not investigated. However, propagation of the virus was reported in porcine transplants in non-human primates.[22,23] It has been shown that transplantations of kidneys from pigs infected with PCMV into non-human primates reduced survival time of the transplant.[22,23] Using kidneys from alpha-1,3-galactosyltransferase knockout (GalT-KO) animals, a survival time up to 53 days was observed in the transplanted baboons, when PCMV was absent, but only 14.1 days when the animals were infected with PCMV.[22] The 53-day survival time was noted when piglets from PCMV-positive sow were delivered by cesarean section, confirming safety of this type of delivery. Although the GöMP breed was also based on cesarean section, PCMV was detected in some animals using highly sensitive methods. In another preclinical trial, cynomolgus monkeys received GalT-KO kidneys from pigs not infected with PCMV and survived for 28.7 days on average, whereas transplants from pigs infected with PCMV survived only for 9.2 days on average,[23] suggesting a direct implication of PCMV on transplant survival in non-human primates.

It is also unclear whether PLHVs pose a risk for pig-to-human xenotransplantation. PLHV-1, PLHV-2 and PLHV-3 are common and latent porcine pathogens.[17] The transmission routes of PLHV in pigs are not well known. It is suggested that PLHV may be transmitted by pre-partum cross-placental vertical transfer and postpartum horizontal transmission; however, cross-placental transfer is not the common way.[19] Between 26% up to 88% of animals in different herds in Germany, Ireland, France, Spain, and the United States were infected with one of the PLHV.[17,24] In contrast to PCMV, early weaning cannot eradicate PHLV.[25] There is evidence that PLHV-1 is associated with post-transplant lymphoproliferative disease (PTLD) in MGH miniature swine following allogeneic haematopoietic stem cell transplantation.[26–28] The clinical symptoms of experimental porcine PTLD are similar to those of human PTLD caused by the Epstein-Barr virus (human herpesvirus 4, HHV-4).

The present study is relevant for two main achievements: First, we detected PCMV (and previously HEV[11]) in animals produced under spf conditions, indicating that better testing and elimination has to be performed when introducing animals into spf facilities.

Second, newly developed highly sensitive PCR methods (system 2)[16] allow detection and quantification of PCMV in animals which have been tested negative by less sensitive PCR methods (see Table 4). To prevent virus transmission during xenotransplantation, such highly sensitive methods are required.

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DISCLOSURE

We disclose that the authors AR and UB are affiliated with Beta-O_2 Technologies, a biomedical company developing an implantable bio-artificial pancreas for treatment for patients with diabetes. The device is designed for human transplants, but could be used also with pig islet cells.

AUTHORS' CONTRIBUTIONS

VM and EP performed the experiments, collected and analyzed the data. AR and UB contributed to organization of the experiments and critical reading of article. VM and JD carried out the concept design and were involved in data analysis/interpretation. VM and JD were drafting the article.

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