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Research Article

Active Replication of Porcine Cytomegalovirus (PCMV) Following Transplantation of a Pig Heart into a Baboon despite Undetected Virus in the Donor Pig

Vladimir A. Morozov[1#]*, Jan-Michael Abicht[2], Bruno Reichart[3], Tanja Mayr[2], Sonja Guethoff[3,4], and Joachim Denner[1#]

[1]Robert Koch Institute, HIV and other Retroviruses, Germany
[2]Department of Anesthesiology, Ludwig-Maximilians-Universität München, Germany
[3]Walter Brendel Centre of Experimental Medicine, Ludwig-Maximilians-Universität München, Germany
[4]Department of Cardiac Surgery, Ludwig-Maximilians-Universität München, Germany
[#]Both authors contributed equally

*Corresponding author
Joachim Denner, Vladimir Morozov, Robert Koch Institute, HIV and other Retroviruses, Nordufer 20, D-13353 Berlin, Germany, Email: DennerJ@rki.de, MorozovV@rki.de

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Abstract

Viral zoonoses may represent a serious problem when xenotransplantation using pig cells, tissues, and organs will be performed. Recently we reported multiorgan failure, mainly liver failure, in a baboon (Papio anubis) after orthotopic transplantation of a heart from a genetically modified pig and showed that the baboon was not infected with hepatitis E virus (HEV). Since pigs are frequently infected with porcine cytomegalovirus (PCMV), and the infection is latent and therefore difficult to detect, screening for PCMV was performed, using a highly sensitive real-time PCR and a nested PCR. In addition, to confirm the absence of HEV, more specific immunological methods and a real-time PCR were used. Only two out of six available pigs, including the selected donor animal, were found negative for both viruses. Nevertheless, PCMV was detected in the blood of the baboon. The titre of PCMV in the blood was above 3x104/ml and analysis of its sequence confirmed that it was PCMV, but not baboon CMV. The data highlight a considerable risk of PCMV infection and call for regular screening of donor pigs applying the most sensitive detection methods.

ABBREVIATIONS

DPF: Designated Pathogen-Free; HEV: Hepatitis E Virus; IgG: Immunoglobulin G: PCMV: Porcine Cytomegalovirus; Spf: Specified-Pathogen Free; PLHV: Porcine Lymphotropic Herpes viruses.

INTRODUCTION

The shortage of human donor organs for transplantation has limited the treatment of patients with severe organ failure. As an alternative, xenotransplantation was developed, using pig cells, tissues and organs. The pig has attracted interest as transplant donor because of its size, partial physiological compatibility, breeding characteristics and the potential for genetic modifications. To overcome the hyperacute rejection induced by preformed anti-Gal antibodies and other immunological rejection processes, numerous genetically modified pigs were created to express human complement regulatory or other proteins and/or α 1,3-galactosyltransferase knockouts(GalT-KO) (for review see [1].

Transplantation of pig cells, tissues and organs may be associated with transmission of porcine microorganisms, including viruses [2]. Whereas most porcine microorganisms with a zoonotic potential such as hepatitis virus E (HEV), porcine cytomegalovirus (PCMV), and porcine lymphotropic herpes viruses (PLHV-1, -2, -3) may be eliminated by designated pathogen-free (DPF) breeding of the animals [3-5], porcine endogenous retroviruses (PERVs) cannot be eliminated because they are part of the genome of all pigs [6]. However, the potential risk posed by all these viruses for the human transplant recipient is still unknown. Here we report transmission of PCMV by orthotopic heart transplant from a genetically modified pig which was tested PCMV negative. Like in previously published

cases PCMV may be involved in the earlier transplant rejection and death of the recipient baboon [7,8].

PCMV belongs to the genus Roseolovirus in the subfamily Betaherpesvirinae in the Herpesviridae family [9]. The enveloped viral particles are 150–200 nm in diameter and the only one completely sequenced PCMV contain a 128,367 bp linear double-stranded DNA genome with 79 open reading frames [9]. Pigs are frequently infected with PCMV. The virus causes generally mild disease in animals younger than six weeks. Infected adult pigs may not present with symptoms of disease and the virus remains latent and therefore difficult to detect. The primary site of virus replication in pigs is in the nasal mucous glands or the lachrymal glands and the virus can be recovered from nasal and ocular discharge [10]. Since the related human cytomegalovirus (HCMV) is an important cause of morbidity and mortality in human allotransplant recipients [11], it may be expected that PCMV has a similar effect. It is important to note that PCMV as Roseolovirus is closer related to the human herpes virus 6 (HHV-6) and HHV-7, than to HCMV (HHV-5). In two studies it has been shown that PCMV induced a significantly reduced transplant survival when PCMV-infected kidneys were transplanted in two non-human primate species, baboons and cynomolgus monkeys [7,8]. In another case, in addition to the transmission of PCMV the baboon cytomegalovirus was activated also in the recipient [12], however, in all these cases it remained unclear, whether PCMV was able to infect cells of the recipient.

Recently we reported a multi organ failure in a baboon after orthotopic transplantation of a heart from a triple genetically modified pig and showed that the baboon was not infected with HEV [13]. Here we concentrated for the above mentioned reasons on PCMV. In addition, we confirmed the absence of HEV in the transplanted baboon by more sensitive methods in comparison to the first analysis.

MATERIALS AND METHODS

Animals and treatment

Six candidate pigs (*Sus scrofa*) were available for transplantation. They were all triple genetically modified, all crossbreds of German Landrace and Large White, with a GalT-KO, expressing human CD46 and human thrombomodulin (Revivicor, Blacksburg, VA, USA and Institute for Molecular Animal Breeding and Biotechnology, Faculty of Veterinary Medicine, LMU, Munich, Germany). They were analysed for virus infections. The transplant recipient, a 4 years old male baboon, was treated with anti-CD20mAb (19 mg/kg, rituximab, Roche, Basle, Switzerland, days -7, 0, +7, +14), anti-CD40mAb (50 mg/kg , 2C10R4, Mass Biologics, Boston, MA, USA, days -1, 0, +3, +7, +10, +14, +19, later weekly), and anti-thymocyte globulin (ATG, 5 mg/kg, Fresenius/ Neovii Biotech, Gräfelfing, Germany, days -2 and -1). Continuous immunosuppressive treatment included mycophenolate mofetil (40mg/kg/d, CellCept, Roche Pharma AG, Basle, Switzerland) and methylprednisolone (10 mg/kg/d, urbason, Sanofi-Aventis, Paris, France). Immunosuppression was performed as described by Mohiuddin et al. [14,15], with slight modifications. Prophylactic anti-herpes virus and anti-bacterial treatment was performed using ganciclovir (5mg/kg/d, given intravenously, Cymeven, Roche Pharma AG, Basel, Switzerland) and cefuroxim (50mg/kg/d, Fresenius, Bad Homburg, Germany, until day 5) given during the entire experiment. The animal experiment was approved by the Goverment of Upper Bavaria, Munich, Germany (reference number 55.2-1-54-2532-184-2014).

Western blot analysis

To screen for immunoglobulins G (IgG) against HEV, a Western blot analysis was performed using two recombinant HEV proteins as described [16]. First, a recombinant genotype 3 (GT3) ORF 2 - HEV antigen (aa 326-608, GT3-Ctr, 32 kDa) containing the entire immunodominant region and second, a recombinant 44.5 kDa protein with a glutathione-S transferase (GST) tag fused to the ORF2 fragment (aa 452-617, Prospec, Ness Ziona, Israel) (Figure 1A). Electrophoresis of the antigens was performed in precast preparative Tris-Glycine 4%–20% gradient SDS-PAGE gels using SDS Tris-Glycine sample buffer (Novex, Life Technologies Carlsbad, CA, USA). Proteins were transferred for 2 h at 40ºC (46V) onto supported nitrocellulose (GE Healthcare Amersham, Protran, 0. 22 µm, BA83). The membrane was stained with 0.1% Ponceau red to control the efficacy of transfer and cut into strips. The load of antigens per strip was estimated at 300 ng. The membranes were blocked with 6% dry milk in PBS with 0.1% Tween 20 (blocking buffer) overnight at 40ºC. Strips were treated with sera diluted 1:150 in blocking buffer for 2 h at room temperature. Goat anti-pig IgG (Abcam, Cambridge, UK) or goat anti-human IgG alkaline phosphatase conjugated antibodies (Sigma-Aldrich, 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, Madison, WI, USA). Sera from a HEV-infected and non-infected pigs were used as positive and negative controls, respectively.

RNA and DNA isolation

To screen for HEV, RNA was isolated twice from 100 µl of blood and 100 µl sera using RNAeasy Blood and Tissue kit as recommended by the manufacturer (Qiagen GmbH, Hilden, Germany). To screen for PCMV by nested PCR, DNA was extracted from sera and blood of the pigs using two DNA extraction kits: DN easy Blood and Tissue kit (Qiagen GmbH, Hilden, Germany) and ZR viral DNA kit (Zymo Research Corp., Irvine, CA, USA).DNA and RNA were quantified using a NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Worcester, MA, USA).

Nested PCR analysis

To detect PCMV, a nested PCR was performed targeting the DNA polymerase (DNApol) gene using the following pairs of primers for PCR amplification: F1 (nt. 63-81) 5'- ACGGGGATCGACGAGAAAG,), R1 (nt. 412-388) 5'-TCTAGACGACAGGACATTGTTGAT, F2 (182-201) 5'- GAAGAGAAAGGAAGTGAAGG, and R2 (nt. 387-368) 5'- GTCACTCGTCTGCCTAAGC (nt. positions corresponding to GenBank AJ222640) [17]. The reaction was performed using the GoTaq hot start green master mix x2 and the parameters of the PCR were as recommended by the supplier (Promega, Madison, WI, USA). 120 ng of DNA from sera and blood were tested in a 40 cycle PCR using the following parameters: denaturation was at 95ºC for 30 sec, the annealing step at 58ºC for 30 sec and extension at 70ºC for 40 sec. One microliter from the first reaction was taken for the

35 cycles nested PCR using the same denaturation parameters and 54ºC as annealing temperature for 30 sec and extension at 70ºC for 30 sec. The conditions of this extended conventional PCR targeting PCMV DNApol were mostly the same as described above (with exception of the extension time). The following primers were used: F1 ext. (nt.1695-1717,GenBank AJ222642)5'-GGACAGCAGAGAAGGGTGTTCAC-3' and R1 ext. (nt.2276-2254, GenBank AJ222642)5'-GTTGATAAAGTCACTCGTCTGCC-3', the annealing temperature was 54ºC and extension was carried outat 70ºC for 45 sec.

The detection limit of the conventional one round PCR was 15 copies, that of the extended conventional PCR - 25 copies and the limit of the nested PCR were 5 copies, as estimated using serial dilution of the reference plasmid with a known copy number.

Real-time PCR analysis

To screen for HEV, a real-time RT-PCR targeting the orf 2 of the HEV was performed using primers, a probe and parameters as described for a diagnostic real-time RT-PCR system [18].

To screen for PCMV, a real-time PCR with a detection limit of 2-5 copies was performed using SensiFast probe no ROX one-step kit according to supplier recommendations (Bioline GmbH, Germany). 60 ng of DNA were used for testing. Reaction mixture contained 400 nM of both primers Fr-t (nt. 279-297) 5'- AATGCGTTTTACAACTTCACG and Rr-t (nt. 382-361) 5'-CTGAGCATGTCCCGCCCTAT, 100 nM of the probe (nt. 331-350), 5'- 6FAM-CTCTAGCGGCGTCCATCACC-BHQ in a final volume of 20 µl. The following conditions for amplification were used: denaturation at 95ºC for 5 min and 45 cycles of amplification with denaturation at 95ºC for 10 sec, annealing at 59ºC for 20 sec and extension at 60ºC for 25 sec. A standard curve was generated using serial dilutions of a reference plasmid with a known copy numbers [17]. Reporter fluorescence was measured using an Mx3005P Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA).

Cloning and sequencing

After electrophoresis on 1.2% agarose gel the amplicons were extracted using Invisorb Spin DNA extraction kit (STRATECmolecular, Berlin, Germany). The fragments were ligated into a pCR2.1-TOPO vector according to the protocol of the supplier (Invitrogen Life Technologies, USA). Z cells (Zymo Research, Irvine, CA USA) were transformed with the constructs for 25 min on ice and plated on LB agar/ampicillin dishes for 18 hours at 37ºC. Five colonies were collected and amplified in LB/ampicillin medium overnight at 37ºC. Plasmids were isolated using Pure Yield plasmid miniprep system (Promega, Madison, WI, USA) and cleaved with EcoR1 to check for the inserts. The clones and amplicons were sequenced in both directions using primers from the cloning kit and BigDye terminator v.3.1sequencing kit (Applied Biosystems, Germany).

Alignment and phylogenetic analysis

The Blast program (NCBI) was used for database search. The sequence alignments and neighbour-joining phylogenetic analyses were performed using software package Lasergene Version 10 (DNASTAR, Inc. Madison, USA).

RESULTS

Selection of the donor pig

The donor pig (#6) was selected for transplantation from six available pigs based on the finding that it was negative for the HEV genome,as reported [13]. Blood and sera from these six pigs were tested not only for HEV, but also for a PCMV infection. Screening for PCMV was performed using two newly established PCR diagnostic systems, a nested PCR and a real-time PCR [17]. Detection limits of the systems were 5 and 2 genome equivalents per reaction, respectively.

To detect HEV, RNA isolated from blood was analysed by real-time RT-PCRThree animals (#1-#3)of sixwere found positive for HEV andthree (including donor pig #6) were negative (Table 1). To confirm the previous results, in addition a Western blot analysis was performed using not one, but two recombinant HEV proteins as described [16]. A strong IgG response against two capsid protein of HEV was detected in sera of four animals (Figure 1A). It should be noted, that due to the age (22 days) of thepiglets#5 and #6 Table (1) their IgG immune response against

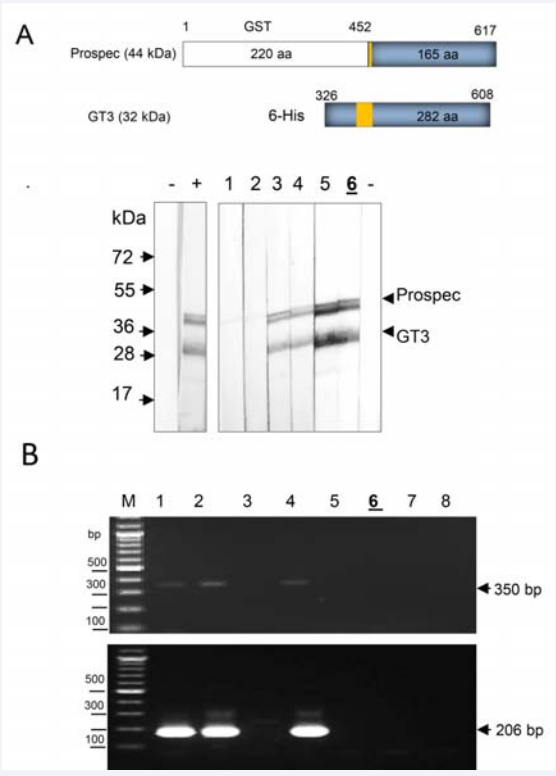

Figure 1 Selection of the donor pig for orthotopic pig-to-baboon heart transplantation: Screening for HEV and PCMV in pigs 45 days before transplantation. A, Western blot analysis of the pig sera using two overlapping recombinant antigens (Prospec and GT3) corresponding to the core antigen of HEV (marked in blue, numbers of amino acids are indicated). The immunodominant region is marked yellow. Animal sera were tested at dilution 1:100. "+" - serum from a HEV infected pig. " –" - serum from non-infected pig, GST, Glutathione S transferase Tag, His, Histidine Tag. B, Nested PCR targeting the DNApol region of the PCMV genome. DNA from sera of animals 1 to 6 was tested. Upper panel is the first PCR round; lower panel is the nested PCR. Positions of the amplicons are given by arrow heads. The donor animal used for transplantation is in bold.

Table 1: Analysis of 6 genetically modified pigs and the baboon recipient for PCMV and HEV infections.

Animal	Sex	Age of the animal at day of sampling	PCMV nested PCR sera	PCMV nested PCR blood	PCMV real-time PCR, (ge/reaction)	HEV real-time RT-PCR (ge/reaction)	HEV Western blot analysis
Pig 1	F	60 days	++	++	+ (2-4)	+ (>100)	Neg.
Pig 2	F	60 days	++	++	+ (2-4)	+ (<10)	Neg.
Pig 3	M	55 days	Neg.	Neg.	Neg.	+ (>100)	+
Pig 4	F	55 days	++	++	+ (10)	Neg.	+
Pig 5	M	22 days	Neg.	Neg.	Neg.	Neg.	+*
Pig 6	M	22 days	Neg.	Neg.	Neg.	Neg.	+*
Baboon	M	4 years	+	+	+ (1.5x 10^3)	Neg.	Neg.

*Likely maternal antibodies; ** ge-genome equivalents

HEV was considered to be maternal. Such vertical transmissions of anti-HEV IgG from mother to offspring were reported [16]. DNA isolated from blood and sera of pigs was tested for PCMV using nested and real-time PCR. Three pigs (#1,#2,#4) of six were found positive in both tests (Figure 1B, Table 1). However, as shown by real-time PCR the virus titre in the infected animals was very low.Results of the genome equivalent estimation based on the serial titration of the reference are shown (Table 1). The pig #6 was negative for HEV and PCMV and therefore selected as donor animal. Although HEV and PCMV were detected in some of the available donor pigs, none of the animals showed clinical signs

Outcome of the transplantation

The heart from pig #6 (#3701 in [13]) was transplanted orthotopically into a baboon using a heart-lung machine as reported [13]. Despite the successful heart transplantation, the recipient baboon died 29 days after transplantation applying a non-toxic, especially non-liver toxic immunosuppression after a short history of liver failure. At day 28 the animal had 901 U/l alanine aminotransferase (ALT), 58 ng/ml troponin I and 1.1 mg/dl bilirubin [13]. It is worth mentioning that the normal ALT level in humans and baboons is 10-40 U/l. More clinical and laboratory data are given in [13].

Lack of HEV transmission

Since a severe liver failure was observed in the recipient baboon, blood and sera from the animal were previously screened for HEV infection using a real-time RT-PCR and a Western blot analysis using a recombinant HEV GT3 protein as antigen [16]. Both tests were negative. Knowing that the level of HEV expression may be very low, the real-time RT-PCR analysis was repeated using newly extracted RNA from sera. The sample from donor pig #6 was found negative again (Table 1). The Western blot was also repeated, but in addition to the HEV GT3 protein another recombinant HEV ORF2proteinwas used (Figure 1A). Serum from donor pig reacted with both antigens, but no antibodies were found in the serum of the recipient baboon (not shown).Thus, the negative result ruling out a HEV infection of the recipient was confirmed.

Detection of PCMV in the baboon

DNA extracted from blood and serum from the baboon was tested for PCMV using recently established nested PCR and real-time PCR diagnostic systems [17]. PCMV was detected already after the first step of conventional nested PCR indicating a virus load >100 genome equivalents per reaction (Figure 2A). Further on a real-time PCR quantification demonstrated that there were 3 x 10^4 genome equivalents/ml blood of the recipient (Table 1). To confirm the porcine origin of the detected virus the amplicon obtained by conventional nested PCR was cloned, and sequenced. Neighbour-joining phylogenetic analysis of the viral sequences detected in the baboon indicated that they belong to the PCMV variant found in pigs from Germany (GenBank acc.AF258039), but not to the CMV from the Cercopithecidae family (Figure 2B).

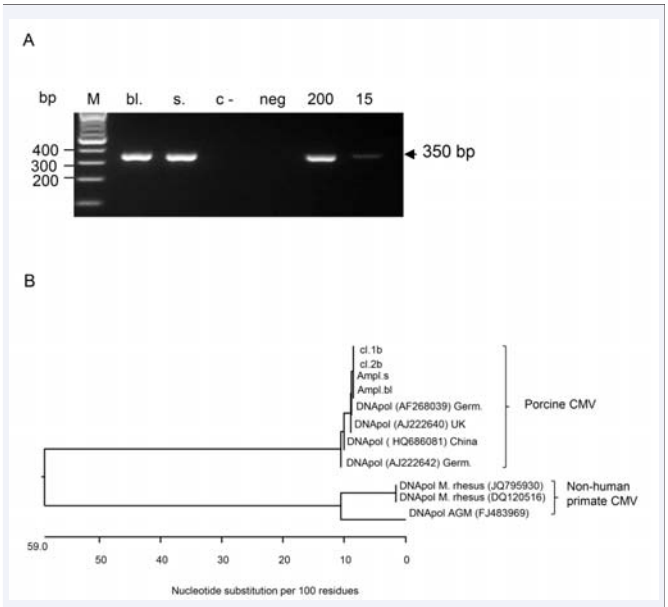

Figure 2 Detection of PCMV in the blood (bl.) and serum (s.) of the baboon after orthotopic pig-to-baboon transplantation of the heart of pig 6 by conventional PCR.A negative DNA control (c-) and a water control (neg) were included. To estimate the copy number of PMCV present in the animal, a dilution of the plasmid was tested (200 and 15 copies). B, Neighbour-joining phylogenetic analysis of the 350bp PCMV DNA polymerase sequence detected in the transplanted baboon. Two cloned sequences (cl. 1b and cl. 2b) and direct amplicons from sera (Ampl. s) and blood (Ampl. bl) were compared with published sequences from different PCMV and viruses isolated from rhesus monkeys (M. rhesus) and African green monkeys (AGM), the corresponding GenBank accession numbers are shown.

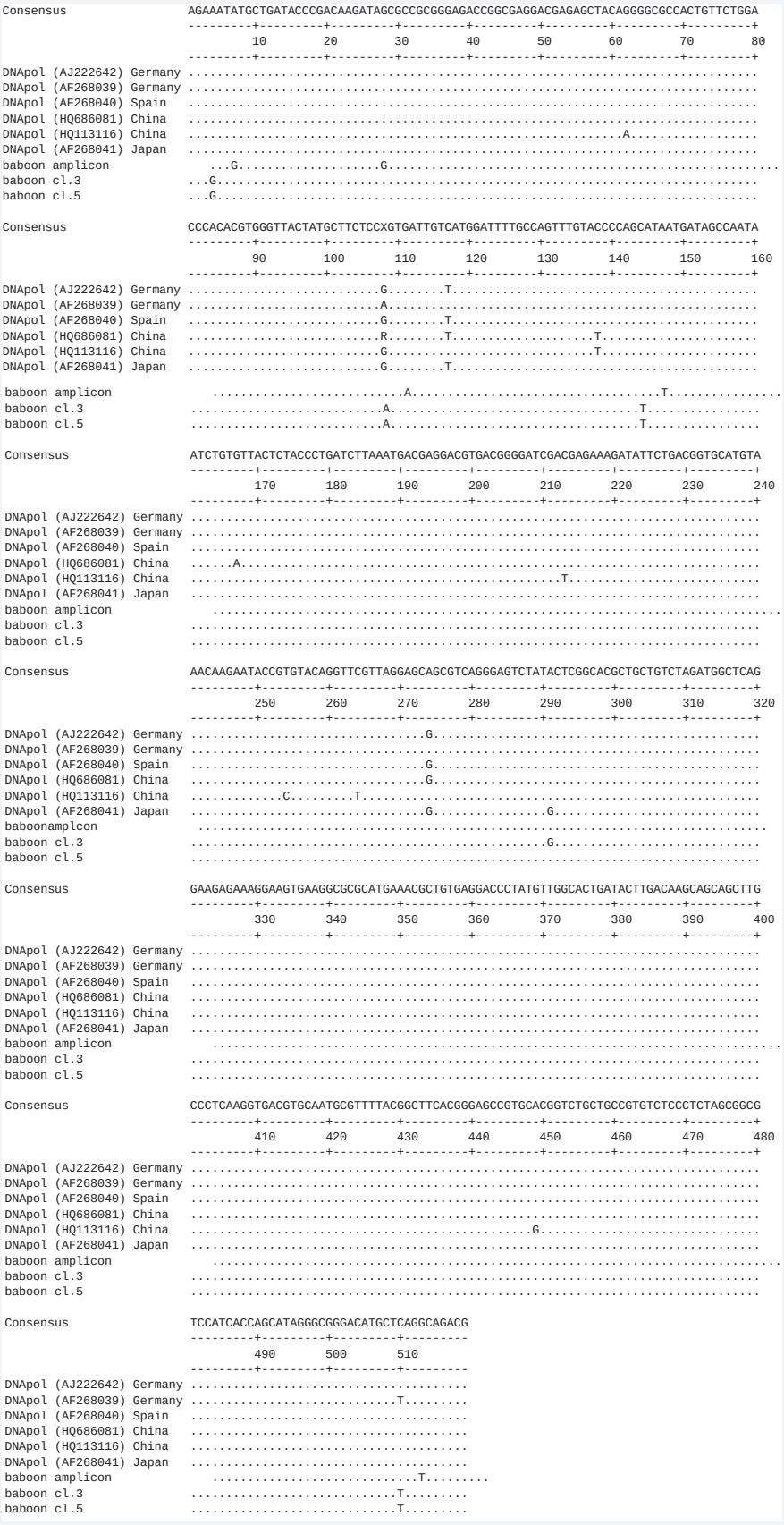

Figure 3 Alignment of an extended PCMV fragment detected in the blood of the transplanted baboon with corresponding PCMV DNApol sequences from the GenBank. In addition to the amplicon, two clones (cl. 3 and cl.5) were sequenced. The acccession numbers are given in brackets and the countries of theorigin are indicated.

As shown, the sequences of the DNApol regions of PCMV and CMV from Cercopithecoidea family are sub-divided into two distinct clusters and that was well supported by bootstrap analysis. In fact, the PCMV detected in the transplanted baboon showed only 42% of nucleotide identity with CMV from the Cercopithecoidea family. To confirm that the detected sequence was not the result of a cross-contamination of the baboon sample with a reference plasmid used in the laboratory, another conventional PCR was established which generated a 580 bp amplicon of the DNApol gene. Amplification was performed; the amplicon was cloned and sequenced. The results proved that the animal was infected with PCMV and revealed mismatches with the reference plasmid indicating that the sample was not cross-contaminated (Figure 3).

DISCUSSION

Here we demonstrate that a baboon recipient of a pig heart was infected with PCMV as a consequence of the xenotransplant, although the donor pig was virus negative when blood was tested using sensitive PCR methods. Obviously PCMV was replicating in the donor pig at a very low level and replication was considerably enhanced in the immunosuppressed recipient baboon.

Although the donor pig was a triple genetically modified animal with a GalT-KO and expressing hCD46 and human thrombomodulin, and although it was found negative when tested for PCMV using ultrasensitive detection methods, the baboon recipient died 29 days after transplantation. The immunosuppression applied was non liver toxic. Ganciclovir was given as a standard procedure, but it did not interfere with virus replication. This correlates well with previous data showing that ganciclovir which is highly effective in inhibition the replication of HCMV, is less effective against PCMV [19]. It remains unclear whether the PCMV infected baboon cells or whether its replication was restricted to the transplanted pig organ. The species-restriction of PCMV is under discussion. Whereas some authors were unable to infect human cells with PCMV [20], others demonstrated infection and expression of viral proteins in primary human fibroblasts [21]. We did not test organs from the deceased animal by PCR-based methods because these methods will not allow discriminating whether PCMV is associated with pig cell or baboon cells. The only method which could discriminate is immunohistochemistry; however there are no PCMV-specific antibodies available.

Although this is only a case report, the picture observed here is in line with previous reports demonstrating a reduced survival time of pig kidney transplants which were derived from PCMV positive donor pigs [10,11]. In the first report, kidneys from GalT-KO animals were transplanted into baboons [11]. The animals had a survival time of 48.3 days when PCMV was absent, and 14.1 days when the kidney came from animals infected with PCMV. When kidneys were taken from pigs which had been derived from PCMV-positive pigs by Caesarean delivery, a survival time of even 53 days was obtained, indicating that Caesarean delivery is an effective strategy to remove PCMV from the pig herd. In the second report, cynomolgus monkeys received GalT-KO kidneys from pigs from two different sources, one source was the Massachusetts General Hospital (MGH)-Nippon Institute for Biological Sciences (NIBS), the other source was the Meji University [10]. Whereas the kidney from the MGH-NIBS pigs survived for 28.7 days on average, the transplants from the Meji pigs survived on average only for 9.2 days. The main difference between these two groups was the presence of PCMV in the Meji pigs, indicating the important influence of PCMV on transplant survival time. Thus, infection with PCMV may pose a significant risk for the human recipients after receiving pig cells, tissues or organs, especially when the recipient is treated with pharmaceutical immunosuppression. The mechanisms how PCMV caused an earlier organ failure were not investigated in details and remain unclear.

To our knowledge this is the first case showing replication of PCMV in a non-human primate that received a transplant from a pig that was considered PCMV-negative after examination of blood and serum using highly sensitive PCR methods.

The main lesson learned from this case is that PCMV may be not detected by a sensitive PCR in the DNA from blood and sera of donor pigs produced for xenotransplantation, and thus, it may pose a serious problem for the transplant recipient. Since the sensitivity of the PCR systems used in this study were close to PCR detection limit of one copy, thus, PCR detection cannot be significantly improved in future. However, the problem of virus detection can be partially solved by examination of different biological samples collected by non-invasive or low-invasive methods (e.g., skin, urine, feces, anal swabs, saliva). These samples could be then tested using established diagnostic PCR systems. Preferentially, this screening should be performed soon after birth. Early testing is very important, since the data could be much more informative, than testing during fettering or later on in life, when the PCMV titer in blood is close or below the detection limits. It is known, that isolation of virus-negative animals is problematic. However, selected animals should be separated from the rest of the herd to form a “clean group” and re-tested shortly before the transplantation. If all animals in the herd are PCMV-positive, virus elimination should begin with Caesarean delivery [10,22], or early weaning [22,24]. In both cases the animals have to be isolated in order to prevent *de novo* infection. Such elimination programs have been suggested [4].

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