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Multiplex PCR for the simultaneous detection of pseudorabies virus, porcine cytomegalovirus, and porcine circovirus in pigs

Chul-Seung Lee [a], Hyoung-Joon Moon [a], Jeong-Sun Yang [a], Seong-Jun Park [a], Dae-Sub Song [b], Bo-Kyu Kang [b], Bong-Kyun Park [a,*]

[a] *Department of Veterinary Medicine Virology Laborotory, College of Veterinary Medicine and BK21 Program for Veterinary Science, Seoul National University, Seoul 151-742, Republic of Korea*
[b] *Research Unit, Green Cross Veterinary Products, Yong-In 449-903, Republic of Korea*

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Abstract

The use of porcine organs is being developed as a means to alleviate the shortage of human organs for transplantation. Recommendations have been published for the microbiological specifications of organ-source pigs to reduce the possibility of a microorganism from pigs being inadvertently transferred to the recipient of the xenograft. The pseudorabies virus (PRV), porcine cytomegalovirus (PCMV), and porcine circovirus (PCV) are infectious agents in pigs that are considered to be of significance for the microbiological safety of xenotransplantation. A multiplex polymerase chain reaction (mPCR) was developed to detect and differentiate among PRV, PCMV, and PCV. The sensitivities of the multiplex PCR were $10^{2.5}$ $TCID_{50}$/ml for PRV, $10^{1.8}$ $TCID_{50}$/ml for PCMV, and $10^{1.8}$ $TCID_{50}$/ml for PCV. The lowest viral concentrations detected by single PCR were $10^{1.5}$ $TCID_{50}$/ml for PRV, $10^{1.0}$ $TCID_{50}$/ml for PCMV, and $10^{1.4}$ $TCID_{50}$/ml for PCV2. Non-specific reactions were not observed when other viruses, bacteria, and Vero cells were used to assess the multiplex PCR. The multiplex PCR was effective in detecting various combinations of one or more of these viruses in pig specimens collected for xenotransplantation.

Keywords: Polymerase chain reaction; Pseudorabies virus; Porcine cytomegalovirus; Porcine circovirus

1. Introduction

Xenotransplantation is a possible solution to the shortage of tissues for human transplantation. Unfortunately, multiple barriers to the broad application of xenotransplantation currently exist, including the risk of infections (Fishman and Patience, 2004). Swine are most often the source of choice for xenotransplantation (Sachs, 1994). A number of potential swine viral pathogens have been identified, but there is limited information about their risk to humans (Paul et al., 2003). Enhanced replication of many pathogens is stimulated by the immune suppression used to prevent graft rejection. As a result, recommendations have been published for the microbiological specifications of organ-source pigs, as well as proposed methods for excluding potentially zoonotic infectious agents (Onions et al., 2000). The technical feasibility of producing and monitoring healthy, qualified pathogen-free (QPF) pigs as xenograft-source animals that are free of significant exogenous infectious agents has been reported (Tucker et al., 2002). Viral pathogens in potential xenograft-source pigs must be identified. This report describes a multiplex PCR (mPCR) assay for detecting exogenous porcine viruses including pseudorabies virus (PRV), porcine cytomegalovirus (PCMV), and porcine circovirus (PCV).

PRV is a member of the Alphaherpesvirinae subfamily of the family Herpesviridae (Pejsak and Truszczyński, 2006). PRV-infected pigs show nervous signs or respiratory signs with high mortality. PCMV is a beta-herpesvirus belonging to the family Herpesviridae. In susceptible herds, the virus may cause fetal and piglet death, runting, rhinitis, and pneumonia. Herpesviruses are the prototype viruses that are activated during immunosuppression (Mueller and Fishman, 2004). Activation of latent herpesvirus infection during periods of intensified immune reactivity to grafts is an important problem for human allotransplantation (Fishman and Rubin, 1998).

* Corresponding author. Tel.: +82 2 880 1255.
E-mail address: parkx026@snu.ac.kr (B.-K. Park).

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PCV is a member of the genus *Circovirus* in the family Circoviridae. Two genotypes of PCV, PCV type I (PCVl) that was originally identified in the porcine kidney (PK15) cell line (ATCC-CCL31) (Tischer et al., 1982) and PCV type II (PCV2) that is associated with post-weaning multisystemic wasting syndrome (PMWS), have been recognized (Nayar et al., 1997). A review of the recommended microbiological specifications for potential xenograft-source pigs has highlighted the possible significance of recently characterized exogenous porcine viruses including PCV (Onions et al., 2000).

The potential for swine viruses to infect humans needs to be assessed *in vitro* and *in vivo* and rapid and more reliable diagnostic methods need to be developed to assure a safe supply of porcine tissues and cells for xenotransplantation. All of the viruses in this study have the potential of being transferred as infectious agents from the donor to the recipient. The objectives of this study are to develop and identify various combinations of the three swine DNA viruses, PRV, PCMV, and PCV, for xenotransplantation research.

2. Materials and methods

2.1. Cells and viruses

The continuous Vero cell line (ATCC-CCL-81) was regularly maintained in Dulbecco's Modified Eagle's Medium (DMEM, WelGENE Inc.) supplemented with 5% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). The virulent PRV Yangsan (YS) strain was provided by the National Veterinary Research and Quarantine Service of Korea. PRV titration was carried out using a 96-well microplate with Vero cells. Determining the $TCID_{50}$ of PRV was performed as described by Gray (1999). The PCV2 DR673 strain was used. The titer (50% tissue culture infective dose, $TCID_{50}$) of DR673 was $10^{5.0}$ $TCID_{50}$/ml and the quantitation method was based on a previous report (Yang et al., 2003).

2.2. Extraction of viral genomic DNA

Cell lysis buffer (500 μl), containing 27% sucrose, saline sodium citrate (15 mM trisodium citrate and 0.15 M NaCl, pH 7.0), 1 mM ethylene diaminetetraacetic acid (EDTA), 1% sodium dodecyl sulphate, and 200 μl/ml proteinase K, was mixed with 200 μl of sample solution and the mixture was thoroughly vortexed and incubated for 1 h at 65 °C. The extraction of DNA was performed as previously described (Yang et al., 2003).

2.3. PCMV and PCV2 quantitation

PRV was serially diluted 10-fold ($10^{5.5}$ to $10^{2.5}$ $TCID_{50}$/ml). Unknown titers of PCMV (OF1 strain) and PCV2 were also serially diluted 10- to 10^5-fold. Viral genomic DNA from each dilution was extracted as described previously and amplified by PCR. PCR products were separated on a 1.5% agarose (Sigma) gel precast with ethidium bromide (0.5 μg/ml) in 1× Tris borate EDTA (TBE) buffer. An image of the gel bands was obtained with Gel Doc and the densities of the amplified DNA bands were measured with a Quantity One quantitation analysis software package (Gel Doc XR, Gel Documentation System, Biorad).

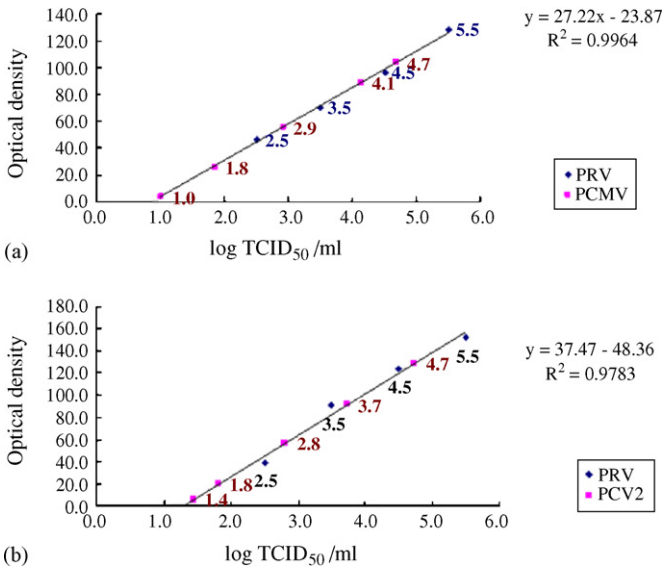

Fig. 1. Quantitation of the viruses against pseudorabies virus: (a) quantitation of PCMV and (b) quantitation of PCV2.

The resulting relative OD values for PRV were compared with those for PCMV to deduce the amount of PCMV DNA. The OD values of the PRV PCR bands were used to construct a standard curve. The OD values of the bands increased in proportion to band size although the number of DNA fragments were the same. Each OD value for the PCMV bands was corrected by PRV band size/PCMV band size (294/413). The quantity of PCMV DNA was calculated from the PRV standard curve using each band intensity as shown in Fig. 1a

The quantitation method for PCV2 was similar to that for PCMV. Each PCV2 OD value was multiplied by the PRV band size/PCV2 band size (294/565). Since PRV is a double-stranded virus and PCV2 is a single-stranded virus, the PCV2 OD values were corrected by multiplying twice. PCV2 DNA was calculated from the standard curve using each band intensity, as shown in Fig. 1b.

2.4. Primer design

Primer pairs specific for PRV gG genes were as in Huang et al. (2004) and primers for detecting PCMV were as in Hamel et al. (1999). Primers specific for both PCV1 and PCV2 were designed on the basis of a PCV open reading frame (ORF) 1 gene. The primer sequences for detection of PCV at same size were obtained using the Primer 3 program (Whitehead Institute/MT Center for Genome Research) with some modifications. PCR primer pairs for each target gene and the amplicon sizes are summarized in Table 1.

2.5. Single PCR

In the single PCRs, a single primer pair was used to detect the target virus. The reaction mixture contained DNA (2 μl), 10× Taq DNA polymerase buffer (2.5 μl), 1.5 mM $MgCl_2$, 2.0 μl