Quantitation of Porcine Cytomegalovirus in Pig Tissues by PCR

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A quantitative-competitive PCR for the quantification of porcine cytomegalovirus (PCMV) was developed. The virus was detected in a variety of pig organs (including potential xenotransplant donations), with viral loads ranging from <10 to 97 genome copies/μg of DNA. This assay will have significant utility for studying the activation and replication of PCMV and in swine models for allo- and xenotransplantation.

Porcine cytomegalovirus (PCMV) is a betaherpesvirus which causes generalized infection in newborn piglets. It has a worldwide distribution and is present in 90% of herds in the United Kingdom, many of which are from well-managed, high-health-status farms (3). Epidemiological features of the virus include evidence of latency and crossplacental infection. Xenotransplantation has the potential to relieve the current donor organ shortage associated with human allotransplantation, and for both practical and ethical reasons, the pig has become the most likely source of such tissues. As clinical trials of xenotransplantation are beginning, concerns have been raised about the potential transmission of porcine organisms to immunocompromised human recipients of porcine tissues (1, 4, 5). As with allotransplantation, there are potential risks associated with the transmission of porcine viruses through xenograft tissues. Although transmission to humans has not been demonstrated in vivo, porcine endogenous retroviruses have been shown to replicate in human cells in vitro (11, 15). Human cytomegalovirus (HCMV) has a variety of direct and indirect effects in the allograft recipient (6). Given the high frequency of seropositivity of swine for PCMV and the efficiency of transmission of HCMV from donor to transplant recipient (6), PCMV may represent another potential risk to humans.

At present the laboratory detection of PCMV is carried out by serology or qualitative PCR (8, 13, 14). We now describe the development of a quantitative-competitive PCR (QC-PCR) assay and its application to the measurement of viral load in porcine tissues. The QC-PCR is based on the coamplification of the test sample with an internal competitor, which differs from the wild-type sequence by the presence of a restriction endonuclease site in the middle of the amplicon. To our knowledge this is the first quantitative PCR assay for the quantification of PCMV to be reported.

The qualitative and QC-PCR assays amplify a region of the polymerase gene (14). Primers PCMV1F (5′ CCTATGTTTG GACTGATACTTGGAC 3′) and PCMV1R (5′ CCCTGAAAA CACTGATACTTGAC 3′) were used in one PCR, and primers PCMV2F (5′ AAGCAGACGGCCCTAAGGTT 3′) and PCMV2R (5′ AACGTGCAATGCGTTTACGGCTTC 3′) were used in the nested assay. The amplicon was then cloned into the pGEM-T Easy vector (Promega), and then used as the template for amplification with primers PCMVF1 and PCMVFM (5′ TAAGCATGTCCTCCGG GCTATGCTGG 3′) were used in one PCR, and primers PCMV1R and PCMVRM (5′ CCAGCATAGCCGAGGAC TGTATTA 3′) were used in the other. The products of these PCR experiments were purified by Wizard PCR Prep (Promega), mixed in equimolar amounts, heated to 95°C for 12 min, and then cooled to room temperature over a 30-min period. The annealed products were 3′ extended using Klenow polymerase and deoxynucleoside triphosphates (dNTPs) (both from Promega), and then used as the template for amplification with primers PCMV1F and PCMV1R. The 236-bp PCR product was cloned into the pGEM-T Easy vector, and the mutation was confirmed by restriction endonuclease mapping and DNA sequencing.

For the qualitative nested PCR, the first-round primers PCMV2F (5′ AAGCAGACGGCCCTAAGGTT 3′) and PCMV1R amplify a 212-bp amplicon and the nested primers PCMVFB (5′ ACGTGCAATGCGTTTACGGC 3′) and PCMVFR (5′ ACTTTCTCTGACAGTATTCTCTAG 3′) amplify a 160-bp product. Three of the primers show 100% nucleotide homology to the DNA polymerase nucleotide sequences of three PCMV strains (55b, B6, and OF-1) deposited in GenBank, suggesting their ability to amplify different isolates. Each PCR mixture contained 100 ng of each primer, 1.5 mM MgCl2 (Bioline), 200 μM each dNTP, 1 U of Taq polymerase (Bioline), and the target DNA, in (NH4)2SO4 PCR buffer (Bioline) to a final volume of 50 μl. The cycle parameters used for the first round were initial denaturation at 95°C for 6 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The final extension was increased to 10 min. In the nested PCR the annealing temperature was reduced to 55°C. Using plasmid dilution experiments the sensitivity of the qualitative PCR assay was <10 genome copies/reaction. The specificity of...
the PCR assay was tested by amplifying DNA from other beta-
herpesviruses: human herpesvirus 6 (HHV-6), HHV-7, and HCMV. None of these samples yielded detectable amplification
products (data not shown).

To assess the utility of the control sequence in the QC-PCR,
a range of known copy numbers of the wild-type and control
plasmids were coamplified. A single round of PCR was per-
formed when 10^0 to 10^6 input copies were used, while a nested
PCR of 15 second-round cycles was used for 10^2 to 10^9 input
copies. PCR conditions and cycle parameters were the same as
those for the qualitative assay. Following amplification, 10 μl
of the amplicons was digested with 10 U of Smal (Boehringer
Mannheim) and separated by polyacrylamide gel electrophore-
sis (PAGE) on a 10% (first round) or 12% (nested round) gel.
The gels were stained with ethidium bromide and photo-
graphed. The intensities of the bands for wild-type and control
amplicons were as previously described (10), and the input
copy number of wild-type sequence was calculated. The
range of quantification for each control sequence copy number
was ±0.5 logunit, i.e., with a control plasmid copy number of
100, between 50 and 500 copies of wild-type plasmid were
tested. The mean result from three experiments was plotted
against the known input copy numbers of wild-type sequence.
Figure 1 shows that the calculated and actual copy numbers of
PCMV were highly correlated (r = 0.998; P < 0.0001 for a
linear fit curve), suggesting that the QC-PCR method was highly
reproducible and could be used to accurately quantify PCMV.

To test the applicability of the qualitative and QC-PCR
assays, five pig tissues (lung, liver, salivary gland, kidney, and
gut) derived from multiple animals from a well-characterized
herd of inbred miniature swine being sacrificed for other on-
going studies (12) were tested for the presence of PCMV. All
animal care and study protocols were approved by the Animal
Studies Committee of the Massachusetts General Hospital.
DNA was extracted by overnight incubation of tissue samples
with proteinase K at 65°C, followed by phenol-chloroform ex-
traction and ethanol precipitation. Qualitative analysis tested 1
μg of extracted DNA, and four of the five tissues were PCR
positive (lung, liver, salivary gland, and kidney). The gut tissue
sample was consistently PCR negative. QC-PCR was then ap-
plied to determine viral loads, utilizing 10 and 100 input copy
numbers of control plasmid. When viral loads were obtained
for a test sample using both input copy numbers of control
sequence, the value used was that where the wild-type-to-
control-amplicon signal ratio was closest to equivalence. The
viral loads in the liver and salivary gland were below the thresh-
old of quantification and were given an arbitrary value of <10
PCMV genome copies/μg of DNA. The kidney contained 38
genome copies/μg of DNA, and the lung contained 97 genome
copies/μg of DNA.

In conclusion, this report describes the development and
application of a QC-PCR assay for the quantification of PCMV,
similar to assays currently used in our laboratory for the quan-
tification of human herpesviruses including HHV-6, HHV-7,
and HCMV (2, 7, 9). This assay will be useful in further studying
the pathogenesis of PCMV and will enable the detection and
accurate quantification of PCMV in porcine organs in pigs
being bred for use in xenotransplantation.

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