Multiply-primed rolling-circle amplification (MPRCA) of PCV2 genomes: Applications on detection, sequencing and virus isolation

Diogenes Dezen a,*, Franciscus Antonius Maria Rijsewijk b, Thais Fumaco Teixeira a, Carine Lidiane Holz c, Samuel Paulo Cibulski a, Ana Cláudia Franco b, Odir Antonio Dellagostin d, Paulo M. Rohe a,b

aLaboratório de Virologia, FEPAGRO Saúde Animal, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Caixa Postal 47, Eldorado do Sul, 92990-000 RS, Brazil
bDepartamento de Microbiologia, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Av. Sarmento Leite 500, Porto Alegre, 90050-170 RS, Brazil
cCIRAD, Département Systèmes Biologiques, UR-15, Campus International de Baillarguet, 34398 Montpellier, France
dCentro de Biotecnologia, Universidade Federal de Pelotas, Pelotas, RS, Brazil

ARTICLE INFO

Article history:
Accepted 19 October 2009
Available online xxxx

Keywords:
Swine
Porcine circovirus type 2
PMWS
Rolling-circle amplification
Infectious copy

ABSTRACT

Multiply-primed rolling-circle amplification (MPRCA) was used to amplify porcine circovirus type 2 (PCV2) genomes isolated from tissues of pigs with signs of post-weaning multisystemic wasting syndrome (PMWS). Two of the amplified PCV2 genomes were cloned in plasmid and sequenced. Both were nearly identical (1767 nt) except for one silent substitution in the region coding for the capsid protein (ORF2). In addition, they showed high nucleotide sequence similarity with PCV2 isolates from other countries (91–99%). To investigate whether the MPRCA amplified PCV2 genomes could be used to produce infectious virus, the cloned genomes were isolated from the plasmids, recircularized and used for transfection in PK-15 cells. This procedure led to the production of infectious virus to titres up to $10^{5.5}$ TCID50/mL. It was concluded that MPRCA is a useful tool to amplify PCV2 genomes aiming at sequencing and virus isolation strategies, where particularly useful is the fact that it allows straightforward construction of PCV2 infectious clones from amplified genomes. However, it was less sensitive than PCR for diagnostic purposes.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Porcine circovirus type 2 (PCV2) is a member of the genus Circovirus of the family Circoviridae (Pringle, 1999). It is closely related to porcine circovirus type 1 (PCV1), a non-pathogenic contaminant detected in the PK-15 cell lineage (Tischer et al., 1982). The overall DNA sequence identity within either PCV1 or PCV2 isolates is greater than 90%, whereas the similarity between PCV1 and PCV2 isolates was reported to vary from 68% to 76% (Hamel et al., 1998). The PCV2 virion comprises a non-enveloped capsid with a diameter of about 17 nm, displaying an icosahedral symmetry and packaging a single-stranded circular DNA genome of about 1.76 kb (Mankertz et al., 2004).

PCV2 is distributed worldwide in swine herds and is a serious cause of economical losses for the pig industry. The virus is regarded as the major infectious agent involved in post-weaning multisystemic wasting syndrome (PMWS), an emerging disease of swine described in the late 90s, characterized by progressive weight loss, respiratory signs and jaundice (Clark, 1997). Macroscopic lesions include granulomatous interstitial pneumonia, lymphadenopathy, granulomatous hepatitis and nephritis (Allan et al., 1998). Other clinical manifestations have also been linked to PCV2 infections, such as porcine dermatitis and nephropathy syndrome (PDNS), an immune-mediated vascular disease affecting the skin and kidney (Smith et al., 1993), reproductive failure (Kim et al., 2004) and the porcine respiratory disease complex (Kim et al., 2003).

In search for a method to detect PCV2 genomes, we employed the so called “multiply-primed rolling-circle amplification” (MPRCA). MPRCA was designed for the isothermal (sometimes referred to as “cold”) amplification of circular DNA templates (Dean et al., 2001). The process is based on random primed amplification of circular DNA by the DNA polymerase of bacteriophage Phi 29 (Dean et al., 2001). By strand displacement synthesis, a high molecular-weight DNA is produced in the form of repeated copies of the template. From these, linearized copies of the full genome can be excised with a restriction enzyme which recognizes a single cleavage site on the viral genome (Rector et al., 2004). The method has been successfully used for the detection of a number of viruses (Rector et al., 2004; Niel et al., 2005; Johne et al., 2006b; Navidad et al., 2008).

Here we describe the use of MPRCA to detect and amplify the full genome of PCV2 isolated from tissues of pigs displaying clinical signs for PMWS and studies to determine the diagnostic value of such a method. In addition, viral DNA amplified by MPRCA was
cloned, sequenced and used in transfections in order to determine whether such DNA would give rise to infectious virus.

2. Materials and methods

2.1. Tissue samples

Ten, eight to twelve week-old pigs displaying clinical signs of PMWS were received from pig farms in the state of Rio Grande do Sul, Brazil. At arrival, pigs were displaying dyspnoea, enlargement of superficial inguinal lymph nodes, pallor, jaundice and diarrhoea. Animals were subjected to necropsy and tissue samples from kidneys, liver, lungs, mesenteric lymph nodes and spleen were collected and stored at −70°C until used.

2.2. DNA extraction and polymerase chain reaction (PCR)

DNA extraction was performed as described elsewhere (Van Engelenburg et al., 1993), modified as follows; 10 mg of tissue were minced and digested for 4 h at 37°C in 1 ml of lysis buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl) containing 0.5% SDS and 0.1 mg proteinase K. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with two volumes of 100% ethanol and kept at 20°C for 1 h. The DNA was washed in 70% ethanol, air dried and resuspended in 100 μl of TE (10 mM Tris, 1 mM EDTA, pH 7.4). For the PCR 100 ng of total DNA extracted were used and the reactions were performed as described previously (Kim et al., 2001).

2.3. Multiply-primed rolling-circle amplification (MPRCA)/restriction enzyme analysis (REA) and cloning

The samples of each tissue were used in multiply-primed rolling-circle amplification (MPRCA) reactions. A MPRCA protocol (Niel et al., 2005) was carried out in 25 μl volumes with 100 ng of the total extracted DNA.

A restriction enzyme analysis (REA) was performed using 5 μl of MPRCA products and 1 U of EcoRI (Invitrogen) or NcoI (NEB) which are expected to cleave the PCV2 - but not PCV1 - genome at a single site. The products obtained from REA were subjected to electrophoresis in a 1% agarose gel, stained with ethidium bromide and visualized on a UV source. Fragments of 1.7 kb, which correspond to the size of PCV2 genome, were purified using a commercial kit (10 mM Tris, 1 mM EDTA, 100 mM NaCl) containing 0.5% SDS and 0.1 mg proteinase K. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with two volumes of 100% ethanol and kept at 20°C until used.

2.4. Nucleotide sequence analysis

The genomes of samples, named 15/5P and 15/23R, were sequenced. Isolated plasmid DNA was purified using the GFX PCR DNA and gel band purification kit (GE Healthcare) and cloned into the EcoRI site of pCR2.1 using TOP10 Escherichia coli cells, as describe elsewhere (Sambrook and Russell, 2001).

2.5. Phylogenetic analyses

The obtained sequences were assembled with SeqMan software (DNASTar Inc) and similarity analysis was performed with NCBI-BLAST (Altschul et al., 1997). The complete genome sequences so generated were aligned with three PCV2 sequences proposed as prototypes for genotypes a, b and c, according to Segalés et al. (2008), available at GenBank. As an outgroup, a PCV1 sequence was included in the alignment (for Accession Nos. see Fig. 2). Sequences were aligned with the ClustalW program within the MEGA3.1 package (Kumar et al., 2004). Phylogenetic analyses of nucleotide sequences were performed using the Neighbor-joining (NJ) method in the MEGA 3.1 software package, based on Kimura two-parameter distance estimation method. Bootstrap resampling was performed for each analysis (1000 replications) and the genomes were classified in PCV2a, PCV2b or PCV2c genotypes, as proposed by the EU consortium on porcine circovirus diseases (Segalés et al., 2008).

2.6. Transfection

The recombinant plasmids were digested with EcoRI and the 1.7 kb fragment was isolated from 0.7% agarose gel using GFX PCR DNA and gel band purification kit (GE Healthcare). The isolated fragment was recircularized using 4 U of T4 DNA ligase (Invitrogen) and then transfected in PCV1-free PK-15 cells. The cells were grown in MEM supplemented with 10% fetal bovine serum (Soralum), non-essential amino acids and glutamine (Gibco). One day before transfection 1.5 × 10⁵ cells were added to each well of a 24 well plate. The cells were transfected with 1 μg of recircularized PCV2 genome and 3 μl of lipofectamine (Invitrogen), following manufacturer’s instructions. Two days after the transfection the plate was frozen at −80°C and the cell lysates were titrated. The virus was detected using a modified immunoperoxidase monolayer assay (IPMA) (Kamps et al., 1994), using a rabbit anti-PCV2-Cap polyclonal serum as a primary antibody and a anti-rabbit IgG (HRP) (Zymed) as a secondary antibody. The anti-PCV2 serum was purchased from the College of Veterinary Medicine, Iowa State University, USA. Its production was described elsewhere (Sorden et al., 1999).

3. Results

3.1. PCR and MPRCA/REA

The presence of PCV2 DNA was confirmed by PCR in all samples. However, in MPRCA followed by digestion with EcoRI, that cuts the PCV2 genome only once, only 27 (54%) out of 50 samples, corresponding to eight out 10 animals, produced a 1.7 kb fragment (Fig. 1). The remaining 23 samples did not give rise to a fragment compatible with the expected size of the PCV2 genome. Thus, successful viral DNA amplification was achieved in 70% of samples from spleen and kidney, in 50% of samples from mesenteric lymph nodes and in 40% of liver and lung samples (Table 2). These results were reassessed by digestion of the same samples with Ncol. Again the same 27/50 samples were digested giving rise to a DNA frag-

<table>
<thead>
<tr>
<th>Table 1 Oligonucleotides used for sequencing of PCV2 genomes from cloned DNA (in plasmid pCR2.1).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>1F</td>
</tr>
<tr>
<td>418F</td>
</tr>
<tr>
<td>1095F</td>
</tr>
<tr>
<td>1286F</td>
</tr>
<tr>
<td>413R</td>
</tr>
<tr>
<td>886R</td>
</tr>
<tr>
<td>1696R</td>
</tr>
<tr>
<td>1768R</td>
</tr>
<tr>
<td>1549R</td>
</tr>
<tr>
<td>M13F</td>
</tr>
<tr>
<td>M13R</td>
</tr>
</tbody>
</table>

ment of the expected size (1.7 kb), whereas the remainder were not. This confirmed that amplification of PCV2 genome was solely achieved on those 54% of the examined samples.

3.2. Cloning and sequencing

The two samples (15/5P and 15/23R) were fully sequenced. Both sequences were 1767 nt long and differed only at nucleotide position 1603 where sample 15/5P has a guanine residue and sample 15/23R has a thymine residue. Both nucleotide sequences showed a genetic organization characteristic of PCV2 with an origin of replication located in the untranslated region, a coding region for the Rep proteins (ORF1) on the sense strand and a coding region for the capsid protein (ORF2) and for a protein associated with apoptosis (ORF3) on the anti-sense strand.

3.3. Phylogenetic analyses

The NCBI-BLAST analysis with the obtained genome sequences revealed that the degree of identity for both vary from 93% to 99% with PCV2 and 67% to 74% with PCV1. The constructed phylogenetic tree grouped the isolates 15/5P and 15/23R in PCV2b genotype (Fig. 2).

3.4. Transfection

The recircularized double-stranded DNA of PCV2 transfected genome gave rise to infectious virus; the supernatant of the culture was able to infect new cell cultures and antigens could easily be visualized through immunostaining with IPMA (Fig. 3). Infectious virus titres obtained with transfected DNA reached $10^{5.3}$ TCID$_{50}$/mL and $10^{5.55}$ TCID$_{50}$/mL for isolates 15/5P and 15/23R, respectively.

4. Discussion and conclusions

MPRCA has been reported as a highly efficient technique, yielding about 20–30 μg of product from as few as 1–10 copies of human genomic DNA (Dean et al., 2002). However, here it was not as sensitive as the PCR, since PCV2 DNA was detected in all samples by PCR, whereas MPRCA was able to detect PCV2 in only 54% of the samples (Table 2). As total DNA was used in the reactions, this low sensitivity may have been due to competition for primers and dNTPs, since MPRCA is based on random amplification and the amount of viral DNA is rather less abundant than genomic DNA in a sample. To ascertain whether viral DNA had not been missed in samples which did not give rise to a DNA fragment of the expected size after restriction enzyme digestion, an in silico restriction enzyme analysis (not shown) was carried out on 528 complete PCV2 genome sequences deposited in GenBank. From those, 96.8% have a single site for EcoRI, whereas 98.5% have a single restriction site for NcoI. Therefore, no additional restriction sites for those enzymes were to be expected in the samples examined here. Thus, the failure of MPRCA to detect viral DNA on those was not related to the addition of extra restriction enzyme sites; it seems more likely that MPRCA was just not sensitive enough to achieve that. Nevertheless, despite its relatively low sensitivity in comparison to the PCR employed here, MPRCA allowed recovery of the full PCV2 genome from at least one tissue from 8 out of

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>PCR</th>
<th>MPRCA/REA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kd</td>
<td>Li</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Total (%) 100 100 100 100 100 100 70 40 40 50 70 54
the 10 animals tested (Table 2). This may be highly advantageous, particularly if sequencing of the full genome is required - as it has usually been the case when small DNA genomes are searched for. MPRCA was shown to bear additional advantages over usual PCR amplification, since it does not require thermocyclers for amplification and no equipment other than a water bath or incubator at 30 °C is necessary, bringing down the costs of the procedure (Dean et al., 2001). In addition, MPRCA is performed in a single step, allowing detection of viral DNA even with no previous knowledge of the target sequence. It has, though, the disadvantage of requiring further restriction enzyme cutting (Johne et al., 2006a).

Perhaps the greatest advantage of MPRCA over other amplification methods is that it facilitates enormously the process of construction of PCV2 infectious clones from amplified genomes. The abundance of viral DNA generated makes cloning quite straightforward. The transfection of recircularized double-stranded PCV2 DNA gave rise to infectious virus to titres of up to $10^{5.5}$ TCID$_{50}$/mL, confirming the infectious nature of the cloned genomes and the capacity of the viral progeny obtained to generate fully infectious PCV2 virions. However, the direct transfection of MPRCA products from infected tissues does not guarantee that there is only one isolate in the sample. As the PCV2 genomic DNAs were recovered from cloned material, it is possible to ensure that no other adventitious contamination was introduced. Other authors have used carried out transfections with the digested material directly from the MPRCA; however, this might lead to the presence of more than one isolate in the transfection mix (Ferreira et al., 2008). In addition, the method used here allowed a rapid recovery of infectious virus. PCV2 propagation in cell culture by conventional methods can be time consuming and rarely gives rise to infectious titres equal to or greater than $10^{3.0}$ TCID$_{50}$/mL (Zhu et al., 2007).

The approach employed here could be used as an alternative method for virus isolation and propagation.

In the present study, MPRCA was less sensitive than the PCR employed here for diagnostic purposes. However, it was highly useful to amplify full length PCV2 genomes from tissues of infected pig tissues, with the advantage that MPRCA does not require thermal cycling and gives rise to full genome copies. Such full length genomic DNA can provide nucleotide sequences of high quality and may also be used for straightforward generation of infectious PCV2 clones.

The results of the phylogenetic analyses revealed that the two samples from which PCV2 genomes were sequenced (15/5 and 15/23) can be assigned to the PCV2b genotype, according to the classification scheme proposed by Segalés et al. (2008). Recently published data on Brazilian PCV2 sequences reported PCV2b as the most frequently genotype detected amongst Brazilian PCV2 genomes (Ciacci-Zanella et al., 2009).

Conflict of interest statement

None of the authors of this paper has financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of the present paper.

Acknowledgements

We thank Dr. David Driemeier (UFRGS) for providing tissues of swine with PMWS. Financial support: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Financiadora de Estudos e Projetos (FINEP). DD was in receipt of a Master’s grant from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). PMR is a CNPq 1C research fellow and FAMR is a CNPq visiting scientist.

References


Smith, W.J., Thompson, J.R., Done, S., 1993. Dermatitis/nephropathy syndrome of pigs. The Veterinary Record 132, 47.


