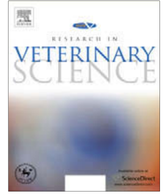
Research in Veterinary Science 89 (2010) 133–139

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc



Detection and genotyping of porcine circovirus in naturally infected pigs by oligo-microarray

Yonghou Jiang [a,*], Hanwu Shang [b], Hui Xu [c], Xianfeng Ding [a], Lingyan Zhao [c], Li Fang [d], Weijie Chen [c]

[a] College of Life Sciences, Zhejiang Sci-Tech University, Hangzhou, PR China
[b] College of Life Sciences, China Jiliang University, Hangzhou, PR China
[c] Centre of Animal Disease Control of Zhejiang Province, Hangzhou, PR China
[d] College of Animal Sciences, Zhejiang University, Hangzhou, PR China

ARTICLE INFO

Article history:
Accepted 14 January 2010

Keywords:
Porcine circovirus
Microarray
Oligonucleotide probe
Detection
Genotyping

ABSTRACT

A rapid and reliable method for the identification of porcine circovirus (PCV) genotypes based on oligonucleotide microarray hybridization has been developed. The genotype-specific oligonucleotides (22–30 mer) immobilized on the surface of glass slides were selected to bind to the multiple target sites within the replication gene that are conserved among individual PCV genotypes. Cy5-labeled DNA targets were amplified in a PCR with primers common to both genotypes. The identification of PCV genotype was based on hybridization with several individual genotype-specific oligonucleotides. This approach combines the high sensitivity of PCR with the selectivity of DNA–DNA hybridization. The utility and feasibility of oligonucleotide microarray hybridization was evaluated by testing standard and 87 clinical isolates. Analysis of the specimens showed that this microarray-based method is capable of unambiguous identification of both genotypes and fivefold more sensitive than gel electrophoresis. Our results indicated that the oligonucleotide array is useful for the identification and discrimination of PCV from clinical isolates and specimens in a clinical laboratory.

© 2010 Elsevier Ltd. All rights reserved.

1. Introduction

Porcine circovirus (PCV) is a 17 nm in diameter and circular single-stranded DNA virus with a genome of 1.7 kb. PCV was classified into the genus *Circovirus*, the family *Circoviridae*, (Index of Viruses – Circoviridae, 2006). Two types of PCV have been detected and characterized, and were subsequently named PCV type 1 (PCV1) and PCV type 2 (PCV2) (Allan et al., 1995). PCV1 has been recognized as a contaminant in a porcine cell line (PK-15) for over 25 years and considered nonpathogenic (Tischer et al., 1986 and Allan et al., 1998). PCV2 has been identified as an agent consistently associated with postweaning multisystemic wasting syndrome (Allan et al., 1998 and Choi et al., 2000).

Postweaning multisystemic wasting syndrome (PMWS) is an emerging swine disease first described in Canada in 1991. Typically, morbidity rates can reach 5–50% in affected herds, and mortality is close to 100% in pigs that develop the full spectrum of symptoms associated with the disease (Morozov et al., 1998). The rapid and simultaneous emergence of the disease in many different parts of the world as well as the uncertain mode of transmission and high rates of mortality in pigs suffering from PMWS has caused great concern throughout the swine industry. Even though PCV1 and PCV2 seem to be very different from a pathogenic point of view, both viruses are very similar at the genomic level since they have about 80% overall nucleotide sequence identity (Meehan et al., 1998). A technique for identification and differentiation of the nonpathogenic and pathogenic strains is needed since serological surveys have demonstrated that infection with PCV is very common in pig herds (Allan et al., 1998; Tischer et al., 1986). Furthermore, since pig has been considered as potential donors of cells, tissues and organs for human xenotransplantation and used as a bioreactor for producing therapeutic proteins, detection of PCV contamination is essential to prevent the risk of endogenous PCV transmission to human recipient (Frankish, 2002; Paleyanda et al., 1997).

Currently, commonly used methods for detecting and genotyping of PCV include in situ hybridization (ISH) (Calsamiglia et al., 2002; Kim and Chae, 2002), polymerase chain reaction (PCR) using type-specific primer (Allan et al., 1999), multiplex PCR (Ouardani et al., 1999; Huang et al., 2004), as well as PCR combined with restriction fragment length polymorphism (RFLP) analysis (Fenaux et al., 2000). However, the ISH is less sensitive and RFLP is time-consuming and laborious compared to PCR (Calsamiglia et al., 2002; Jang et al., 2004).

Although PCR amplification followed by separation and characterization of DNA products by gel electrophoresis is a simple and sensitive method, this approach has inherent shortcomings. Highly

* Corresponding author. Tel.: +86 86843197; fax: +86 86843196.
E-mail address: yonghoujiang@163.com (Y. Jiang).

0034-5288/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rvsc.2010.01.009

sensitive PCR amplification tends to generate more non-specific DNA products, which complicate interpretation of results. Furthermore, in cases where multiplex PCR is used for the simultaneous detection of several genetic markers, these non-specific products may be a significant problem (Volokhov et al., 2002).

A combination of PCR with DNA–DNA hybridization instead of gel electrophoresis significantly improves the specificity of target sequence detection in the presence of non-specific PCR products (Chizhikov et al., 2002). Microarray technology provides an ideal solution to this problem. DNA and oligonucleotide microarrays are widely used for detection and genotyping of microorganisms (Uttamchandania et al., 2009).

The aim of the present study was to establish a microarray using type-specific oligonucleotide as capture probes, which allow the identification and differentiation of PCV1 and PCV2. The practicability and specificity of the DNA microarray for the identification and differentiation of PCV1 and PCV2 in pig population was evaluated with reference strains and clinical specimens.

2. Materials and methods

2.1. Virus strains and sample

PCV1-infected cells (HZ2006, EF533941) were kindly provided by Professor Zhou Jiyong (Zhejiang University). PCV2 reference strain (HZ0202, AY217743) and negative controls, bovine viral diarrhea virus (BVDV) (Oregon C24 strain), transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV) vaccine (Harbin Weike Biotechnology Development Co., Catalogue Number: 030718), pseudorabies virus (PRV) vaccine (China Animal Husbandry Industry Co., Catalogue Number: 0412014–3), porcine reproductive and respiratory syndrome virus (PRRSV) (ZJ2005, DQ269472), classical swine fever virus (CSFV) (Hangzhou strain) and porcine parvovirus (PPV) vaccine (Beijing Zhonghai Animal Health Science and Technology Co., Catalogue Number: 0040401), were all kept in our laboratory. Eighty-seven clinical specimens including lymph nodes, tonsils, lungs, as well as spleens were obtained from Zhejiang Veterinary bureau, collected from 73 4–8-week-old sick or dead piglets in 11 local farms and 14 aborted fetuses from different abortion cases during the period from June 2005 to September 2006.

2.2. PCR primers and probes

Sequences of PCV1, PCV2 and other virus were retrieved from GenBank in September 2005. Multiple alignments of 84 PCV2 and 22 PCV1 whole sequences were made using Clustal W software (DNAStar, Inc., Madison, USA) and the region containing conserved as well as variable sequences was chosen as diagnostic region which allowed us to design species and genotype-specific probes. Specificity at the probe design level was provided by selecting oligonucleotide probes that are perfect matches (i.e. identical in complementary sequence) to their intended targets differing significantly compared with another sequence, and have a narrow range of melting temperature (T_m). Based on the following criteria: probe length range between 22 and 30 nucleotides, melting temperature range of 65–75 °C, and two or more mismatches with homologous sequences in another genotype, probes were designed by Primer Premier 5.0 software (Premier Biosoft International, California, USA). Two sets of upstream primer and downstream primers common to both PCV1 and PCV2 and one set specific for PCV1 for validation of results were designed by Primer Premier 5.0 according to conserved sequences of diagnostic regions. Primers demonstrated no deleterious secondary structures, and no significant identity ($E < 1.0$) to non-PCV sequences was found by a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). A single sequence based on potato 18S rRNA with a complementary Cy5-labeled sequence spiked in hybridization solution was used as a positive and served as a direct indication of whether or not the hybridization conditions were adequate and also as a spatial marker for ease of viewing. Two probes originated from two plant viruses, Cucumber mosaic virus and tobacco mosaic virus, with no identity to the target sequences, were chosen as negative controls. The uniqueness of the sequences of the probes designed from the PCV diagnostic region was analyzed with the BLAST search. The 5′ end of each probe was modified by adding poly T_{10} and an aminolink group to enable covalent immobilizing on the aldehyde-coated glass surface (Baiao, Shanghai, China). The complete list of oligonucleotide primers, probes and their characteristics are listed in Table 1.

2.3. Cloning of target fragments

To provide stable and quantified templates for microarray hybridization experiment, the amplified fragments were cloned to plasmid. Briefly, viral genomic DNA was extracted either from 200 μl of lysates of PK-15 cells infected with each virus strain, or 200 mg of fresh or frozen clinical specimens with a commercial DNA extraction kit (Sangon, Shanghai, China) according to the manufacturer's direction. The extracted DNA was amplified by PCR. PCR was carried out in a 50 μl volume consisting of 2 μl of virus DNA, 2.5 U Taq polymerase (TaKaRa, Dalian, China), 1 × PCR buffer, 2 mM $MgCl_2$, 200 nM species-specific primer pairs, and 200 mM each dATP, dCTP, dGTP, and dTTP. The PCR program consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 30 s, with a final extension step of 72 °C for 10 min. The PCR product was DNA sequencing (Sangon, Shanghai, China) and separated by gel electrophoresis and purified with a PCR clean-up kit (V-gene, Hangzhou, China). The purified PCR product was ligated into the pGEM-T Easy vector (Promega, Madison, USA). The recombinants were transformed into competent *Escherichia coli* DH5α cells. Several colonies were selected at random and the plasmids were extracted using the Rapid Plasmid Daily-prep kit (V-gene, Hangzhou, China) according to the manufacturer's instructions and verified by PCR and DNA sequencing (Sangon, Shanghai, China). The DNA concentration of the recombinant plasmids was spectrophotometrically determined (Molecular Devices, USA).

2.4. Labeling of target

Labeled amplification reactions (50 μl) were conducted as above protocol except 10 nM dCTP, 4 nM Cy5-dCTP (Amersham Bioscience, USA) instead of 200 mM dCTP, and 2 μl of recombinant plasmids instead of 2 μl of virus DNA. The PCR product was precipitated with 0.1 V of 3 M NaAc and two volumes of ice-cold absolute ethanol for at least 30 min at −20 °C. Finally, after air drying, precipitated labeled product was resuspended in 20 μl of 1 × hybridization buffer (Baiao, China) for the following hybridization. Internal positive control was prepared simultaneously.

2.5. Microarray fabrication

Microarrays were printed on aldehyde-coated glass slides (2.5 cm by 7.6 cm) (Baiao, Shanghai, China) using a microspotting device (BioDot, Inc., CA) with a single pin delivering approximately 20 nl of a spotting mixture per spot. The final spotting mixture contained 10 μM concentration of probe in a spotting buffer containing 3 × SSC (1 × SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0). Each oligonucleotide probe was spotted in triplicate.

A range of 70–75% humidity was maintained in the humidity chamber of the device to prevent rapid evaporation of spotted

Download English Version:

https://daneshyari.com/en/article/2455723

Download Persian Version:

https://daneshyari.com/article/2455723

Daneshyari.com