Journal of Virological Methods 191 (2013) 1–8

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

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

Comparative evaluation of conventional polymerase chain reaction (PCR), with loop-mediated isothermal amplification and SYBR green I-based real-time PCR for the quantitation of porcine circovirus-1 DNA in contaminated samples destined for vaccine production

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Article history:
Received 9 October 2012
Received in revised form 12 March 2013
Accepted 14 March 2013
Available online 26 March 2013

Keywords:
Porcine circovirus type1
Polymerase chain reaction
Real-time polymerase chain reaction
Loop-mediated isothermal amplification
SYBR green I-mediated polymerase chain reaction

A B S T R A C T

Porcine circovirus type1 (PCV1), described initially as a contaminant of a porcine kidney cell line, is ubiquitous within the swine population The presence of PCV1 in porcine cell lines can lead to contamination during both human and porcine vaccine production. Therefore, a rapid, specific, sensitive and practical method is needed for the detection of PCV1 in bio-products. The aim of this study was to compare three assays in their ability to accurately quantify PCV1 virus in biological samples, namely loop-mediated isothermal amplification (LAMP), SYBR green I-based real-time polymerase chain reaction (PCR) and conventional PCR. All assays yielded successful quantitation of PCV1 DNA and differentiated between PCV1-free and–contaminated cells. In addition, the results were specific for PCV1, since amplification of samples containing closely-related PCV2 or other pathogenic swine viruses yielded negative results. The lowest detection threshold of 10^2 copies was displayed by the SYBR green I-based real-time PCR assay. In addition, this assay was the most effective in detecting PCV1 contamination in a set of commercially available porcine vaccines. Therefore we conclude that SYBR green I–based real-time PCR is specific and sensitive for detecting PCV1 in biological samples and maybe used for quality control of vaccine and biomaterial production.

1. Introduction

The porcine circovirus (PCV), identified initially in 1974 as a non-pathogenic contaminant of the porcine kidney cell line PK-15 (ATCC CCL-33) (Tischer et al., 1982; Tischer et al., 1974), is presently designated as PCV type 1 (PCV1) (Allan et al., 1998). Initial serological studies suggested that PCV1 was ubiquitous in the swine population worldwide but not linked to clinical disease (Allan et al., 1995; Dulac and Afshar, 1989; Tischer et al., 1986). Later, PCV type 2 (PCV2), which is closely related to PCV1, was associated with post-weaning multisystemic wasting syndrome (PMWS) in piglets (Allan and Ellis, 2000), a disease that is spreading worldwide. Both PCV1 and PCV2 have a single-stranded circular DNA genome of about 1.7 kb (Todd et al., 2005) and share 80% nucleotide sequence identity (Olvera et al., 2007).

The PK-15 porcine cell line has been used for the development and production of a number of veterinary and human vaccines (Katayama et al., 1998; Roic et al., 2006). However, the use of cell culture for production of species-specific vaccines, albeit an advance in vaccine development, has the potential for the transmission of viral diseases (Pastoret, 2010). In this regard, since PCV is highly resistant to physicochemical inactivation procedures applied during vaccine production (Allan and Ellis, 2000; Allan et al., 1994; Welch et al., 2006), the use of PK-15 cell line may lead to contamination with PCV. Indeed, the presence of PCV1 DNA has recently been reported in vaccines designed for both human and veterinary use (McClenahan et al., 2011; Victoria et al., 2010). More troublesome is the fact that several human vaccines are found to

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http://dx.doi.org/10.1016/j.jviromet.2013.03.014

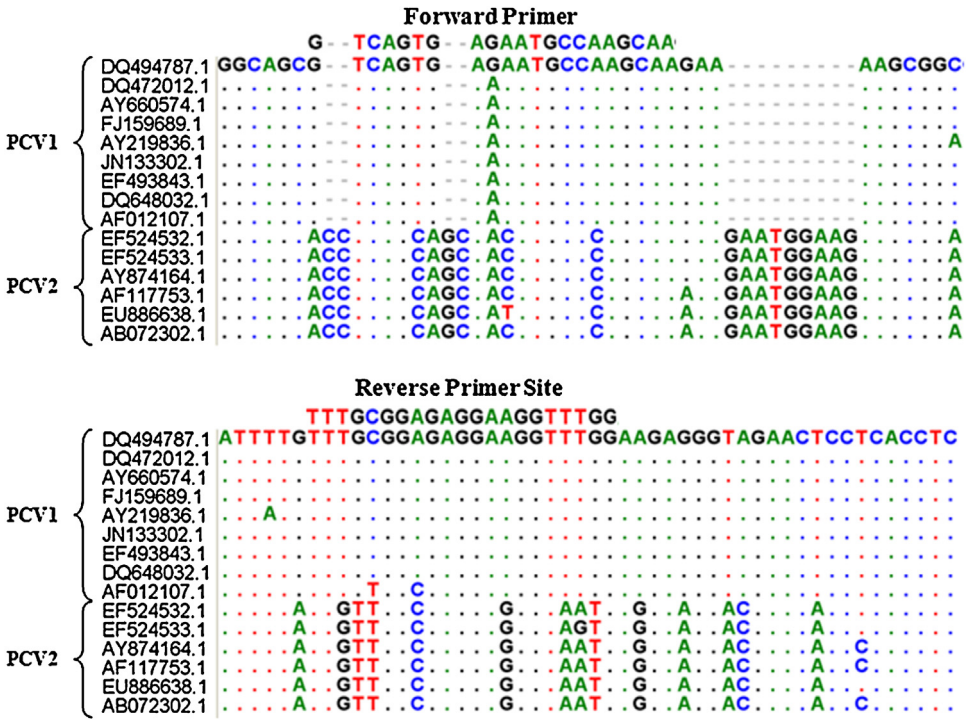

Fig. 1. Alignment of the 152 bp target area used to design the primers used for porcine circovirus type 1 (PCV1) real-time PCR analysis. Clustal W alignment of the PCV genome sequences was downloaded from GenBank (Access No. JN398656.1).

contain full length PCV1 genome with the potential of being infectious [15]. Therefore, it is critical to ensure the use of PCV1-free porcine-derived biological materials in order to prevent the distribution of potentially pathogenic virus during vaccine production.

Ensuring virus-free vaccine production calls for accurate, sensitive and practical methods of viral genome detection. Among the nucleic acid based methodologies, the polymerase chain reaction (PCR) is a sensitive and specific technique that can be performed with relative speed. For the purpose of detecting viral DNA a substantial number of assays have been described based upon variations of the PCR technique (Larochelle et al., 1999; Park et al., 2009; Yue et al., 2009), including multiply primed rolling-circle amplification (Dezen et al., 2010), loop-mediated isothermal amplification (LAMP) (Chen et al., 2008; Zhao et al., 2011), and microarray analyses (Jiang et al., 2010). Real-time PCR has been widely applied due to its high sensitivity and reliability (Mackay, 2004). This method generates a fluorescent signal during the amplification cycle that is directly correlated with the amount of target DNA molecules produced as well as inversely correlated with the number of PCR cycles needed to reach a fluorescent signal threshold (Higuchi et al., 1993). Thus the amount of product generated at each PCR cycle is typically detected using the 5′nuclease assay which is based upon the specific hybridization of a dual-labeled TaqMan probe to the PCR product (Holland et al., 1991). An alternative and cheaper approach is that of the fluorescent dye SYBR green I which binds specifically to double-stranded DNA. A problem with the latter, however, is the potential generation of non-specific double stranded products or primer dimers that will hybridize with the dye and give a false positive reaction. Therefore, the use of specific primers and the optimization of primer concentration are crucial for the accuracy of this method (Ponchel et al., 2003). In addition, LAMP has been developed as a gene amplification technique that is simpler and less expensive than real-time PCR (Notomi et al., 2000). This technique relies on autocycling strand displacement DNA synthesis carried out without the denaturation of DNA templates and therefore at isothermal temperature (Nagamine et al., 2001). Amplified products are visualized as precipitates in a turbid solution with no specific reagent or equipment requirement for the detection of amplified DNA (Mori et al., 2001), thus making this an attractive alternative to more common PCR methods.

Given the importance of defining the best method for detecting viral particles in biological materials destined to vaccine production, the aim of this study was to compare SYBR green I-based real-time PCR, LAMP and conventional PCR for their reliability in detecting and quantifying PCV1 DNA. The goal was to ascertain the most accurate, sensitive and practical method to be applied routinely in the clinical vaccine production setting.

2. Materials and methods

2.1. Cell lines and viruses

PK-15 cells were maintained in Dulbecco's modified Eagle's medium DMEM medium (GIBCO BRL, U.S.) supplemented with 6% fetal bovine serum (FBS; HyClone, U.S.) and antibiotics. The PCV1/JL strain was isolated from a PK-15 cells, which was positive by PCR. PCV2 (a and b) was isolated from pigs with postweaning multisystemic wasting syndrome (PMWS). PCV1 and PCV2 were propagated in PK-15 cells.

2.2. Primer design

Primers for real-time PCR (Fig. 1) and loop mediated isothermal amplification (LAMP) were designed based upon the conserved region of the PCV1 genome sequence (GenBank accession number JN398656.1) using Primer Premier 5 and Primer Explorer V4 software, respectively. An NCBI blast search was performed to determine the oligonucleotide specificity. Primers were synthesized by Invitrogen (Lifetechnologies, Beijing, China) and the corresponding sequences are presented in Table 1.