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Use of PCR-based assays for the detection of the adventitious agent porcine circovirus type 1 (PCV1) in vaccines, and for confirming the identity of cell substrates and viruses used in vaccine production

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A B S T R A C T

Safety and quality are important issues for vaccines. Whereas reversion to virulence poses a safety risk with live attenuated vaccines, the potential for the presence of adventitious agents is also an issue of vaccine quality. The recent detection or porcine circovirus type 1 (PCV1) in human vaccines has further highlighted the importance of quality control in vaccine production. The purpose of this study was to use a novel conventional PCR to detect PCV1, and subsequently screen materials used in the manufacture of vaccines at Bharat Biotech International Limited, India. The genome or gene fragments of PCV1 were not detected in any of the vaccines and materials tested, including the live attenuated rotavirus vaccine candidate ROTAVAC®. Further, the identity of the cells and the viruses used as starting materials in the manufacture of these vaccines was confirmed by species-specific PCR or virus-specific RT-PCR, and no cross-contamination was detected in any case. The methods can be applied for regular in-house quality control screening of raw materials and seeds/banks, as well as formulated vaccines.

1. Introduction

Vaccines must meet strict safety and quality standards. The major factors that affect vaccine safety are reactogenicity and reversion to virulence, the latter factor being a risk associated with live attenuated vaccines. Vaccine quality, on the other hand, depends on multiple factors, the control of which requires highly stringent processes involving detailed characterization of the raw materials, the intermediate products as well as the final bulk and the formulated vaccines. The formulated vaccine must contain sufficient quantities of antigenic material deemed to be protective and efficacious. In addition, the vaccine should not contain any other infectious agent or antigen, and must meet the regulatory guidelines on the acceptable limits of contaminants arising from the substrate as well as the chemicals and reagents used during the bioprocess. In the majority of cases, the preferred method for the production of viral vaccines involves infection of cell substrates, followed by harvesting and purification. Inactivation of the live agent is performed at any stage before the final formulation. The absence or partial inactivation of the agent in the case of live attenuated vaccines, e.g., polio, measles, mumps, rubella, and rotavirus vaccines, leaves the window open for the survival and subsequent propagation of any contaminating (adventitious) agent.

Contamination of vaccines with infectious agents or extraneous materials is not new (Pastoret, 2010). Even under a closed system of production, extraneous agents can enter through the cell substrate, growth supplements, or dissociating agents used in the propagation of cells, or embryonated eggs used for the production of vaccines (Kniazeff et al., 1975; Polak et al., 2008). Simian virus-40 was the earliest recognized adventitious agent, being detected first in polio and then in adenovirus vaccines (Geissler et al., 1985; Hilleman, 1998; Lewis, 1998; Sangar et al., 1999). Assays for the detection of adventitious agents encompass detection of characteristic cytopathic effect (CPE) produced in cultured cells, hemagglutination or hemadsorption, serology to detect proteins expressed by adventitious agents, electron microscopy, and inoculation of the test material into laboratory animals or chick embryos. For most of the assays, the virus strain used to produce the vaccine is first neutralized with specific antibodies, and the neutralized material is used for the testing (FDA, 2010a). However, all these assays are time-consuming. Moreover, at least some adventitious agents remain persistent or latent without causing any visible change *in vitro* or overt clinical symptoms *in vivo*. Thus, nucleic acid-based methodologies, including PCR (Häuptli et al., 1997; Studer

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et al., 2002; Takagi et al., 1996), which are sensitive and specific, and can be performed rapidly, have brought about a considerable change in the detection of adventitious agents. Such assays have led to the identification of contaminants such as endogenous avian leucosis viruses (Barbosa et al., 2008; Fadly et al., 2006; Hussain et al., 2001, 2003; Johnson and Heneine, 2001; Shahabuddin et al., 2001; Tsang et al., 1999), and pestiviruses (Giangaspero et al., 2004; Harasawa, 1995; Kulcsar et al., 2010; Sasaki et al., 1996; Studer et al., 2002) as adventitious agents that compromise vaccine quality.

Porcine circovirus type 1 (PCV1) was discovered originally as a contaminant of the porcine kidney cell line PK15 (Dulac and Afshar, 1989; Tischer et al., 1974, 1982). It belongs to the family *Circoviridae*, and is related very closely to porcine circovirus type 2 (PCV2) as well as to the recently proposed cycloviruses (Li et al., 2010). Whereas PCV1 has not been shown to cause disease (Allan et al., 1995; Fenaux et al., 2003; Tischer et al., 1986), PCV2 is associated with post-weaning multi-systemic wasting syndrome in piglets (Sorden, 2000). Both viruses have a circular single-stranded DNA genome of ~1.7 kb (ICTV, 2009; Tischer et al., 1982) and they share 80% nucleotide identity (Olvera et al., 2007). Since PCV1 does not produce CPE, nor does it exhibit hemagglutination or hemadsorption, the only way to detect contamination with PCV1 is by sensitive immunological or molecular biological assays.

Recently, the application of metagenomics revealed the presence of PCV1 or its nucleic acid in human live rotavirus vaccine Rotarix® (GlaxoSmithKline) or in inactivated polio vaccines (Baylis et al., 2010; McClenahan et al., 2011; Victoria et al., 2010). Indeed, five out of eight live attenuated vaccines screened by this approach were found to contain extraneous viral nucleic acids (Victoria et al., 2010). Similarly, another live attenuated rotavirus vaccine (RotaTeq®, Merck) was found to contain DNA from both PCV1 and PCV2 (CDC, 2010a,b; Victoria et al., 2010). The PCV1 DNA in Rotarix® was detectable even after treatment with nucleases, suggesting that the viral DNA was encapsidated (Victoria et al., 2010). Quantitative PCR analyses have shown that the viral harvest, the purified bulk and the final vaccine formulation contained 10^{10}, 10^{9} and 10^{7} copies of PCV1 DNA/ml, respectively (FDA, 2010b). PCV1 contained in Rotarix® has been demonstrated to infect cells *in vitro* (McClenahan et al., 2011), indicating that the virus is live. Besides human vaccines, several veterinary vaccines have been reported to be contaminated with PCV1 (Pastoret, 2010; Quintana et al., 2006). Thus, PCV1 is an adventitious agent, and can be a concern in the production of live attenuated vaccines (FDA, 2010b).

Bharat Biotech International Limited (BBIL) is a vaccine manufacturer located in Hyderabad, India. BBIL produces and markets vaccines for rabies and H1N1 influenza, and is developing vaccines for Japanese encephalitis and rotaviral diarrhea, all using cell culture substrates. Only the rotavirus vaccine, ROTAVAC®, is live attenuated (Bhan et al., 1993; Bhandari et al., 2009; Cunliffe et al., 1997; Glass et al., 2005). The objective of this study was to develop tests for identity, cross-contamination and extraneous agents in various bioprocess materials used or produced at various steps employed in the manufacturing of viral vaccines by BBIL. In order to verify the presence or absence of PCV1 in cell banks, virus banks, cell culture supplements, trypsin, and bulk antigen or formulated vaccines, a conventional PCR was developed to detect both PCV1 and PCV2. The identities of cell substrates and viruses as well as cross-contamination were also analyzed by PCR and RT-PCR. The results show that none of the tested materials was positive for PCV, and confirmed the identity of the cell banks and the virus materials. These PCR assays should be useful for in-process and quality control analyses used during vaccine manufacturing processes.

Table 1
Details of the cell lines used in this study.

Description	Tissue/cell type	Sample designation
Vero cells	African green monkey kidney	Vero
MDCK cells	Canine kidney	MDCK
MRC5 cells	Human fetal lung (diploid)	MRC5
Hep2C cells	Human laryngeal carcinoma	Hep2C
293IQ cells	Human embryonic kidney	293IQ
PCV1-contaminated PK15 cells	Porcine kidney	PK15-PCV1
PCV-free PK15 cells	Porcine kidney	PK15-FREE
SVBalb cells	Mouse fibroblast	SVBalb

2. Materials and methods

2.1. Cell lines, virus and vaccine samples, cell culture supplements, trypsin, bulk antigen or formulated vaccines, and primers

The details of the cell lines used in this study are shown in Table 1. Vero, Madin-Darby Canine Kidney (MDCK), MRC5 and Hep-2C cells were obtained from Cell Banking Facility of BBIL. PK15 (ATCC CCL-33) and SVBalb cells (Gooding, 1979; Hanke et al., 1991) were a kind gift from Dr. David Johnson, Oregon Health & Science University, USA. 293IQ cells were obtained from Microbix Biosystems Inc., Ontario, Canada. Fetal bovine serum (FBS; Invitrogen BioServices India Pvt. Ltd., Bengaluru, India and Moregate Biotech, Australia), New Born Calf Serum (NBCS; PAA Laboratories Inc., Canada), trypsin–EDTA (Invitrogen BioServices India Pvt. Ltd., Bengaluru, India) and N-tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin (TPCK–trypsin; Sigma–Aldrich, Bengaluru, India) were obtained commercially.

The details of the viruses and the vaccine samples used in this study are shown in Table 2. Samples of ROTAVAC®, INDIRAB®, BIOPOLIO®, *HN-VAC*™ and JENVAC® were obtained from BBIL. Samples of Rotarix® (Glaxo SmithKline), oral poliomyelitis (from Panacea Biotech and Biomed) and Measles–Mumps–Rubella (Serum Institute of India) vaccines, were obtained from the local market. DNA extracted from PK15 cells (ATCC CCL-33) contaminated with PCV1 (PK15-PCV1 DNA) was used as a positive control in all PCR testing of the raw materials and the intermediate as well as the final products. While quality control measures, as part of good manufacturing practices, assure that all the strains represent the virus in question, for each of the viruses, the other viruses served as negative controls. Genomic clones of PCV1 (Beach et al., 2010a,b), PCV2a (Fenaux et al., 2002) and PCV2b (Beach et al., 2010a,b) were used as templates in limit-of-detection analyses performed at Virginia Polytechnic Institute and State University. DNA extracted from a clone of PK15 cells generated by end point dilution to be free of PCV1 and PCV2 (PK15-FREE DNA) (Fenaux et al., 2002) was used as a negative control.

The details of the primers used in this study are shown in Table 3. The PCV- and rotavirus-specific primers were designed by analyzing multiply aligned full genome sequences. The sequence of PCV-specific primers was identical between PCV1 and PCV2. All the other primers were adapted from various publications (Sacramento et al., 1992; Schweiger et al., 1994; Parodi et al., 2002; Cooper et al., 2007; Martin et al., 2007; Sapkal et al., 2007; WHO, 2009). The primers were obtained from Sigma–Aldrich, Bengaluru, India, and Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad, India.

2.2. DNA and RNA extraction, quantification and dilution

DNA was extracted from all the samples following standard protocols (Sambrook et al., 2001). Briefly, 100 μl of samples containing