Research Article

Isolation and Identification of Porcine Circovirus 2 from Cases of Respiratory Disease and Postweaning Multisystemic Wasting Syndrome in Pigs

R. Anoopraj1, Jeny K. John1, Menaka Sethi1, R. Somvanshi2, G. Saikumar3*

1National CSF Referral Laboratory and Swine Disease Laboratory, Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India; 2EBH Laboratory, Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh, India
*Corresponding author: saikumarvri@gmail.com

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ABSTRACT

Porcine circovirus disease (PCVD), an emerging viral disease of economic importance, represents several disease manifestations principally caused by porcine circovirus 2 (PCV2). Prevalence of distinct PCV2 genotypes in pig population necessitates to study their replication potential in cell line in order to establish and compare infectivity and pathogenicity. Virus isolation forms an important aid in PCVD diagnosis, especially when multifactorial origin of disease condition is suspected. In the present study, isolation of PCV2 was accomplished by serially passaging the inoculum prepared from suspected tissue samples up to fourth passage in PK–15 cell line. Further, the confirmation of replication in cell line was done by immunoperoxidase monolayer assay and polymerase chain reaction. Information regarding virus isolation and identification of PCV2 isolates circulating in Indian pigs is scarce. In the present work, PCV2 was successfully isolated in PK–15 cell line and demonstrated their replication in cytoplasm and in nucleus by immunoperoxidase assay. Furthermore, the virus isolation together with PCR assay helped to establish the PCV2 etiology in pneumonia and wasting of pigs encountered in the present study.

INTRODUCTION

Porcine circovirus disease (PCVD) is an important emerging viral disease in India with worldwide occurrence inflicting heavy economic losses to swine industry (Ellis et al., 1999; Sharma and Saikumar, 2010; Meng, 2012; Rajkhowa and Saikumar, 2012; Tico et al., 2013). Circovirus type 2 (PCV2), principal causative agent of PCVDs, is associated with several clinical conditions like PCV2 systemic disease (PCV2-SD) which includes post weaning multisystemic wasting syndrome (PMWS), PCV2 lung disease (PCV2–LD), PCV2 enteric disease (PCV2–ED), PCV2 reproductive disease (PCV2–RD) and porcine dermatitis and nephropathy syndrome (PDNS) (Segales, 2012). Usually, PCVDs affect pigs of 5–18 weeks age with morbidity rate of 4–60% and mortality rate of 4–20% (Segales and Domingo, 2002; Zhu et al., 2007).

PCV2 belongs to the family Circoviridae, genus Circovirus, is a small, icosahedral, non–enveloped, single stranded DNA virus containing a circular genome of 17 nm diameter and with 1767–1768 nucleotides (Hamel et al., 1998; Dupont et al., 2008). The PCV2 genome has three major open reading frames (ORFs). PCV2 ORF1 (945 bp) gene encodes replication proteins Rep and Rep’ involved in virus replication (Mankertz et al., 1997). ORF2 (702 or 705 bp), encodes the major capsid protein (Cap) which is also the main antigenic determinant of the virus (Nawagatigul et al., 2000). Both ORF 1 and ORF 2 together comprise 93 % of the PCV2 genome (Opriessnig et al., 2006). ORF3 encodes a protein that is not essential for PCV2 replication, but involved in PCV2–induced apoptosis (Liu et al., 2005).

PCV2 can be divided into three distinct genotypes, namely PCV2a, PCV2b, and PCV2c, by phylogenetic analysis based on ORF2 gene and complete genome. Infectivity and pathogenicity of these genotypes vary and PCV2b is considered as the most virulent genotype (Olivera et al., 2007). Recently, emergence of new variants of PCV2 has also been reported that evolved through events of mutation and/or recombination (Ramos et al., 2013). Therefore, to establish infectivity and pathogenicity, it is essential to demonstrate their replication potential in vitro.

Diagnosis of PCVDs is accomplished chiefly by clinical signs, characteristic gross and histopathological findings and demonstration of viral nucleic acid or antigen in associated lesions. Though, virus isolation is not a gold standard method for PCVDs diagnosis, it adds confidence in confirmation of diagnosis especially when multiple pathogens are involved. PCV–free PK–15 cell line is considered to be the best cell line for replication of PCV2 and is used widely for virus isolation (Tischer et al., 1987; Zhu et al., 2007). Absence of cytopathic effects after infection necessitates demonstration of viral nucleic acid or protein in the infected cell line to monitor viral replication.


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(Allan and Ellis, 2000). Herein, we report the isolation and identification of PCV2 in PK-15 cell line from cases of PCV2–SD with prominent respiratory involvement and PCV2–SD associated with PMWS.

MATERIALS AND METHOD

Collection of Samples

Tissue samples of lung, lymph node, spleen, tonsil and liver were collected aseptically from animals died of pneumonia and PMWS associated PCV2–SD case with respiratory signs and wasting. Animal died of pneumonia was a suckling piglet of 3 days age, which showed non-collapsed lungs with cranio-lateral and ventral consolidation of right intermediate lobe and severely congested carcass lymph nodes at necropsy, while the animal with PMWS was a grower that showed progressive wasting in spite of normal feed intake. At necropsy this animal revealed confluent consolidation of lung lobes, randomly distributed circumscribed abscesses in right diaphragmatic lobe and severely congested carcass lymph nodes. Samples were transported on ice to the laboratory and stored at −80°C till further processing.

PCV2 Detection

DNA was isolated from collected samples of lung, lymph node and spleen of each case by using QIAmp DNA Mini and Blood Mini kit (Qiagen, USA) following manufacturer’s instructions. Initial screening was performed by PCR targeting a fragment of 481 bp size corresponding to ORF2 region of PCV2 (Ellis et al., 1999).

Virus Isolation

Twenty four hours grown, 50–80% confluent PCV–free PK–15 cell cultures were used for inoculation with infected samples. The procedure for PCV2 isolation was adopted as described by Wellenberg et al., (2000) with minor modifications and is outlined as follows. Inoculum for infection of cell line was prepared and consisted of pooled sample of lung, lymph node and liver of necropsy no. 89A–13 and 192A–13. Five hours after inoculation of PK–15 cell line with the prepared inoculum, semi–confluent monolayer was treated with 300 mM D–glucosamine–HCl and incubated for 30 min followed by washing twice with DMEM to remove the D-glucosamine–HCl and then incubation in DMEM containing 10% FCS and 0.5% antibiotic mix for further 48 hrs at 37°C in a 5% CO2 incubator. Negative controls were processed in the same way. After incubation period, cell cultures were subjected to freeze–thaw cycles three times (frozen at −70°C for one hour followed by thawing). The resultant cell lysate was centrifuged at 2,500 rpm for three times (frozen at −70°C) and then incubation in 72°C for 1 min followed by 40 cycles of 95°C for 4 min, followed by 40 cycles of 95°C for 30s, 62°C for 40s, 72°C for 1 min, and final extension step at 72°C for 7 min. DNA extracted from control cells served as negative control template, while a known positive (192A–13, lymph node) sample served as positive control. After electrophoresis, the amplified products were viewed under UV imaging system.

RESULTS

PCV2 Detection

During initial screening, twelve cases were found positive for PCV2 by PCR. Samples from two positive cases (Necropsy no. 89A–13 and 192A–13) were selected for virus isolation based on relative freshness of the sample.

Virus Isolation

After inoculation with the samples, the cells exhibited a slow growth compared to the negative uninfected control cells. No cytopathic effect was observed. Morphologically, there were no differences in the infected and uninfected cells, except for the presence of mild to moderate cytoplasmic vacuolation in the infected cells after fourth passage (Figure 1). The virus could be successfully isolated.
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Indirect Immunoperoxidase Monolayer Assay (IPMA) and PCR

Indirect immunoperoxidase test of PCV2 infected PK-15 monolayer using porcine circovirus 2 antiviral anti-serum revealed both intracytoplasmic and intranuclear positive reaction (Figures 2, 3). In a few cells, positive reactions were present in the entire nucleus of the cell. The replication of virus was further confirmed by PCR reaction with the specific primers which showed positive band of expected size of 765 bp for both cell culture supernatant and cell sediment DNA (Figure 4).

DISCUSSION

PCV2 is ubiquitous in nature. The virus can be recovered from both healthy as well as diseased animals (Jantafong et al., 2011). Therefore, the diagnosis PCVDs requires additional information from clinical signs and associated lesions and mere PCR positivity cannot establish the disease condition. However, PCR is a rapid and reliable technique to detect PCV2 infections (clinical and subclinical) in pigs (Alarcon et al., 2011). Thus, in our study, we chose PCR as detection method to identify PCV2 infections.

Virus isolation is the most reliable method to establish an infection of viral etiology and is considered as the cornerstone in laboratory diagnosis of viral infections (Storch, 2000). In the present study, PCV2 was successfully isolated in PK-15 cell line after infecting the cells with a pooled inoculum of lung, lymph node and spleen from two PCR positive cases and thus confirmed a PCV2 etiology in the disease condition. Treatment of PK-15 cell line with 300 mM D-glucosamine-HCl is recommended after infection with PCV2 to enhance viral replication. This is because, D-glucosamine-HCl enables PCV2 to enter nucleus of PK-15 cells, wherein the viruses utilise the cellular enzymes expressed in S-phase of cell cycle for their replication (Tischer et al., 1987). However, prolonged treatment with D-glucosamine-HCl can lead to cellular damage due to its toxic effects on cell cultures (Allan and Ellis, 2000). After inoculation, the infected cell line showed a slow rate of growth compared to negative control. Virus replication in cell line did not produce any cytopathic effect. This is consistent with previous studies that described similar
findings (Wellenberg et al., 2000; Rajkhowa, 2008). A positive indirect immunoperoxidase test indicated the replication of PCV2 in cell culture. The staining pattern in the infected cells was similar to the pattern described by Allan et al. (1998). Identification of intracytoplasmic and intranuclear staining revealed the presence of viral antigen in both cell compartments. In addition, a positive PCV2 specific amplification of DNA isolated from fourth passage cells and supernatant by PCR further underlined PCV2 replication in cell line. Though, virus isolation is not considered as the gold standard method for PCVD diagnosis due to absence of cytopathic effect, it adds confidence to the diagnosis of PCVD in combination with IPMA and PCR (Wellenberg et al., 2000; Kim and Chae, 2004). This is particularly important in establishing the role of PCV2 as a pathogen in condition like PCV2–SD, since its clinical manifestation is widely considered as a multifactorial outcome. Moreover, in a study conducted to compare efficiency of various diagnostic methods to detect PCV2, virus isolation was found to be more sensitive than immunohistochemistry and in situ hybridisation for demonstration of the virus (Kim and Chae, 2004). Therefore, the successful isolation of PCV2 in PK–15 cell line in the present study helped in confirming the disease etiology. Likewise, isolation studies pertaining to different genotypes of PCV2 help in identifying the differences in their pathogenicity. This will be much beneficial in identifying the replication potential and infectivity of newly emerging recombinant strains of PCV2.

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CONFLICT OF INTEREST
No conflict of interest to declare.

REFERENCES