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Molecular analysis of Porcine Circovirus Type 2 strains from Uruguay: Evidence for natural occurring recombination

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ABSTRACT

Porcine Circovirus Type 2 (PCV2) is a worldwide distributed virus and is considered an important emerging pathogen related to several distinct disease syndromes in pigs.

Genomic structure consists of three major open reading frames (ORFs). ORF1 (*rep* gene) encodes replication-related proteins, ORF2 (*cap* gene) encodes the capsid protein and ORF3 encodes a protein putatively involved in virus-induced apoptosis.

Based on *cap* gene sequences, PCV2 strains are classified into two main genotypes, PCV2a with five clusters (2A–2E) and PCV2b with three clusters (1A–1C).

According to previous theorical studies, PCV2 strains can eventually undergo intra and inter-genotype recombination, mainly within the *rep* gene. Ever since, several evidences of recombination in the field have been reported and confirmed this hypothesis.

In South America, data regarding molecular characterization of PCV2 strains is still scant. Genotyping studies in the region have concluded that PCV2b is the predominant circulating genotype in the region and till now, no recombinant strains have ever been reported.

In this work we thoroughly characterized at the molecular level Uruguayan PCV2 strains by extensive sequence data analysis. Moreover, recombination software tools were applied to explore and characterize eventual occurrence of natural recombination events.

Two recombinant PCV2 strains were detected in this study, as a consequence of an inter-genotype recombination event between PCV2b-1A and PCV2a-2D, as the major and minor parent, respectively. According to recombination software analysis, in both cases the event occurred within the ORF1.

Herein, extensive viral sequence dataset is provided, including the characterization of the first PCV2 recombinant strains ever reported in South America. Additionally, our results suggested a multi-centered source of PCV2 infection in Uruguay, which probably involved Brazilian and European origins.

1. Introduction

Porcine Circovirus Type 2 (PCV2) is an extensively distributed pathogen considered an important emerging virus related to several distinct disease syndromes in pigs, collectively known as Porcine Circovirus Associated Diseases (PCVAD) (Opriessnig et al., 2007). One of the major syndromes is the Postweaning Multisystemic Wasting Syndrome (PMWS) which has a severe economic impact on swine production and industry worldwide (Chae, 2005). Common clinical signs in this emerging disease include wasting, growth retardation and enlarged lymph nodes (Allan and Ellis, 2000). PCVAD also comprise Porcine Dermatitis and Nephropathy Syndrome (Rosell et al., 2000), Porcine Respiratory Disease Complex (Kim et al., 2003) and reproductive failure (West et al., 1999). In addition, PCV2 has been associated with others swine diseases as exudative epidermitis (EE) (Kim and Chae, 2004; Wattrang et al., 2002), and congenital tremor (Stevenson et al., 2001).

PCV2 belongs to *Circoviridae* family and is a small non-enveloped virus, containing a single-strand ambisense circular DNA genome of about 1.76 kb (Allan and Ellis, 2000). Genomic structure consists of three major open reading frames (ORFs). ORF1 (*rep* gene) encodes replication-related proteins (Mankertz and Hillenbrand, 2001), ORF2 (*cap* gene) encodes the capsid protein which forms the icosahedral capsid (Nawagitgul et al., 2000) and ORF3 encodes a protein putatively involved in virus-induced apoptosis (Liu et al., 2005).

Based on phylogenetic analysis performed with *cap* gene, PCV2 strains are classified into two main genotypes, PCV2a with five clusters (2A–2E) and PCV2b with three clusters (1A–1C) (Olvera et al., 2007; Segalés et al., 2008). A third genotype, PCV2c, was only found in Denmark (Dupont et al., 2008).

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Despite the apparently different pathogenicity of the PCV2 strains, analysis of their genomes of worldwide isolates showed a high level of sequence homology (>93%) among them (Larochelle et al., 2002; Mankertz et al., 2000; Meehan et al., 2001; Olvera et al., 2007). Nevertheless, point mutations together with recombination are important forces of PCV2 evolution that contribute to generate genetic variations between viral genomes.

The eventual existence of PCV2 recombination was evaluated by Olvera et al., 2007, analyzing a large dataset of complete genome PCV2 sequences deposited in GenBank using recombination software. Ever since, several evidences of recombination in the field have been reported to validate this hypothesis (Cadar et al., 2012; Cai et al., 2011, 2012; Cheung, 2009; Gagnon et al., 2010; Hesse et al., 2008; Kim et al., 2009; Ma et al., 2007). Moreover, the co-infection with PCV2 strains of different genotypes in pig population, the precondition for recombination, has also been reported (Cheung, 2009; Zhai et al., 2011).

In South America, data regarding molecular characterization of PCV2 isolates is still scant. Genotyping studies of the virus have been carried out exclusively in Brazil, Argentina, Chile and Uruguay, concluding that PCV2b is the predominant circulating genotype in the region (Noriega et al., 2007; Pereda et al., 2011; Ramos et al., 2012; Ciacci-Zanella et al., 2009). Co-circulation of both main genotypes has been detected only in Brazilian pig population (Ciacci-Zanella et al., 2009; de Castro et al., 2012). Nevertheless, in South America, the coexistence of multiples strains in the same pig has not ever been identified and cases of natural recombination have not ever been reported.

In Uruguay, the first set of PCV2 strains has been identified in 2011 in piglets affected with EE (Ramos et al., 2012). Preliminary sequence analysis revealed that the isolates belonged to PCV2b genotype and were closely related to a set of Brazilian PCV2 strains.

The aim of this work was to thoroughly investigate the genetic heterogeneity of PCV2 strains isolated from pigs of two Uruguayan herds, in order to contribute to shedding light over the molecular epidemiology of this emergent infection in the region.

Herein, we present a comprehensive molecular study of the PCV2 strains detected, analyzing an extensive sequence of the viral genome. Our findings reveal the circulation of two natural intergenotypic PCV2 recombinants for the first time in South America, providing an additional evidence of recombination in the field strongly supported by incongruent phylogenetic trees and data from recombination software analysis. We also report the first complete genome sequence data of PCV2 strains from Uruguay.

2. Materials and methods

2.1. Cases and samples

In this study were included three piglets belonging to two Uruguayan herds. Two of them presented PCV2 associated symptoms and the third was asymptomatic.

One of the clinical cases included in this investigation was previously described Ramos et al. (2012) and corresponded to an animal affected with EE. The second clinical case evaluated corresponded to a piglet with PMWS-associated symptoms including wasting and growth retardation.

Blood samples of piglets were extracted by trained veterinary personnel, and serum was obtained.

2.2. DNA extraction and PCV2 detection

DNA was extracted from 200 μL of serum using a commercial kit (QIAamp DNA Mini Kit QIAGEN) according to manufacturer's instructions.

PCV2 DNA detection of affected EE piglet is published elsewhere (Ramos et al., 2012) and the strain was named PCV2_Uy 1 in this investigation. Remaining two cases included in this study were diagnosed under a different and more sensitive experimental protocol. In order to detect PCV2 in this samples, a small region within *cap* gene of 225-bp was amplified by nested PCR as previously described Kim et al. (2001).

2.3. Amplification of cap and rep gene by PCR

The complete ORF2 sequence of the PCV2 strain isolated from piglet affected with EE was previously obtained (Ramos et al., 2012). In order to amplify the entire viral *cap* gene of remaining cases, a single PCR was carried out using the primer set reported by Fort et al. (2007). PCR amplification were performed in 50 μl reaction volume containing 1X PCR buffer, 2 mM $MgCl_2$, 0.5 μM each primer, 0.2 mM of each dNTP and 1.5 U Taq DNA polymerase (Fermentas, Life Sciences) and 10 μL of DNA. Cycling conditions were: 94 °C for 5 min, followed by 37 cycles of 30 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C and a final extension cycle of 10 min at 72 °C.

In addition, the whole *rep* gene was also amplified by a PCR method according to Liu et al. (2005). The PCR reaction mixture was the same as for the *cap* gene and cycling conditions consisted of an initial step at 94 °C for 5 min, 40 cycles of 45 s at 94 °C, 1 min at 58 °C and 1 min at 72 °C and a final extension at 72 °C for 10 min.

PCR products of the expected size were purified using a commercial kit (NucleoSpin® Extract II, Macherey–Nagel, Germany) and sequenced in both directions by Macrogen Sequencing Service, Korea. To corroborate the nucleotide sequence in each case, PCR reactions were performed and sequenced three times.

2.4. Phylogenetic analysis and genetic typing using cap and rep gene

In order to conduct genotyping studies, the molecular analysis of the three PCV2 strains from Uruguay based on *cap* gene was performed with ORF2 published sequences corresponding to different PCV2 genotypes and subtypes. In addition, seven regional strains were included with the aim to establish the relationship between Uruguayan strains and other South American isolates. Sequence alignment was carried out using Clustal W software and the degree of identity among sequences at nucleotide and amino acid was determined by using BioEdit v. 7.0.5.

Phylogenetic tree was reconstructed based on 702 nucleotide sequence by neighbour-joining method with the Kimura two-parameter (K2P) model as the model of nucleotide substitution using MEGA v 5.0 software. Bootstrap values were determined with 1000 replicates of the dataset and a consensus tree was generated. A PCV1 isolate was included as an out-group (FJ475129).

The molecular characterization of the isolates derived from *rep* gene analysis was carried out by a similar approach to that used for the ORF2 region. ORF1 nucleotide sequences of several PCV2 strains deposited in GenBank were included in the phylogenetic analysis and consensus neighbor-joining tree was constructed on the basis of 945 nucleotide sequence.

2.5. Recombination analysis

The recombination study was performed analyzing the entire PCV2 genome of potential recombinants. The complete nucleotide sequences were obtained by PCR amplification of three overlapping amplicons as previously described Vlasakova et al. (2011). Sequencing results of purified PCR products were spliced and consensus sequences were obtained for the two potential recombinant strains isolated.