



Genetic diversity of Indian porcine circovirus type 2 (PCV2) isolates (2006–2018)

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ABSTRACT

Porcine circovirus type 2 (PCV2) is an emerging viral infection in swine population and results in severe economic loss to piggeries worldwide. The present study was conducted to explore the genetic diversity of PCV2 circulating in swine population of India from 2006–2018. A total of 74 heart, lungs, spleen and lymph nodes collected from different regions in Uttar Pradesh, India were subjected to molecular analysis. For studying genetic diversity, the complete nucleotide and ORF2 sequences of 95 PCV2 including 43 nucleotide sequences from India were used. DNA was extracted from samples and positive samples were subjected to full genome sequencing and phylogenetic analysis was done by maximum likelihood method. Phylogenetic analysis of 40 Indian PCV2 genomes downloaded from GenBank along with three new isolates from the current study based on the complete genome and cap gene together with nucleotide sequences of PCV2 isolates from different countries results in a tree in which Indian isolates clustered in 4 different branches includes PCV2b-1C, PCV2a-2D, PCV 2b-1A/B, PCV 2d-2 recombinant group and two new cluster in which one clustered along with unclassified PCV2 viruses from Indonesia and Croatia. The comparison of ORF2 gene among Indian isolates revealed nucleotide identity ranging from 88.6% to 99.6%, indicating the genetic diversity of PCV2 strains circulating in Indian pig. The present work reports for the first time in India the PCV2-1A/B cluster of 2b genotype and all the Indian isolates available in India from 2006–2018 were used in this analysis.

Keywords: Diversity, Genetic, India, PCV2, Pig

Porcine circovirus type 2 (PCV2) is an emerging viral infection in swine population worldwide and results in severe economic loss to piggeries, viz. worldwide. The PCV2 are divided into five genotypes, viz. PCV2a, PCV2b, PCV2c, PCV2d, PCV2e based on pair-wise sequence comparison (Xiao *et al.* 2015). Again PCV2a is subdivided into 2A-D (four clusters), PCV2b is subdivided into 1A-C (three clusters) and PCV 2d is divided into PCV2d-1 and PCV2d-2 (two clusters) (Xiao *et al.* 2015). PCV2e is a new genotype circulating in USA and China (Davies *et al.* 2016). PCV2a and PCV2b are worldwide in distribution, PCV2c was reported only from Denmark and China (Dupont *et al.* 2008) and PCV2d was reported from 14 countries including India (Xiao *et al.* 2015). In India, PCV2 was reported first in 2005 from swine population and was reported from various places in India. Genotypes prevalent in India are PCV2a, PCV2b, PCV2d and some recombinant types (Sharma and Saikumar 2010, Anoopraj *et al.* 2014). Investigation of PCV2 genotype that is circulating in different regions of India by phylogenetic analysis gives an idea on the epidemiology and thereby finding out the molecular evolution of virus. It also gives information on the vaccine candidate that should be used in India for the control and prevention of further outbreaks. The present

study was conducted to explore the genetic diversity of PCV2 circulating in swine population of India from 2006–2018.

MATERIALS AND METHODS

Sample collection: A total of 74 heart, spleen and lymph nodes were collected from different regions in Uttar Pradesh, India during February 2015–November 2016. The samples were collected on ice for molecular analysis and stored at –80°C until further use.

Data source: Along with the sequences obtained in this current study, the complete nucleotide and ORF2 sequence of 95 PCV2 including 43 nucleotide sequences from India was downloaded from GenBank for studying the genetic diversity of PCV2 circulating in India.

DNA extraction and PCV2 detection: Total viral DNA was extracted from the pooled sample using QIAamp DNA Mini kit (QIAGEN, Germany) according to manufacturer's protocol. The DNA extracted was tested by PCR using the primers PCVLF and PCVLR (Larochelle *et al.* 2000). The positive samples were used for complete genome amplification using published primers (Anoopraj *et al.* 2015) and preceded further for sequencing. The complete genome was amplified in three overlapping fragments of size 661, 765 and 676 bp (Anoopraj *et al.* 2015). PCR reactions were performed in 50 µl reaction volume. Final

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PCR products were subjected to electrophoresis on 1.5% agarose gel and was confirmed with Gel Documentation having ultraviolet imaging system. PCR products were purified using commercial DNA gel extraction kit and sample were sent for sequencing in both directions with respective forward and reverse primers used in the PCR amplification. The truncated sequences from all the three fragments were joined by 'Megalalign' programme of Lasergene V.6 software (DNASTAR, USA) to obtain the PCV2 complete genome.

Phylogenetic analysis based on complete genome as well as ORF2 sequence was performed. Apart from the isolates (3 isolates) from this study, sequences of 92 PCV2 isolates representing different genotypes and clusters from various countries were retrieved from GenBank and used as input sequences. Two PCV1 isolates were incorporated as an out-group sequence. Multiple sequence alignment was carried out using ClustalW programme of MEGA v.6 software. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. Initial tree for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. Evolutionary analyses were conducted in MEGA6.

RESULTS AND DISCUSSION

Characterization of the porcine circovirus 2 identified:

Out of 74 cases which were screened by conventional PCR, 54 were pre-weaned piglets between 0–6 weeks and 20 were post-weaned animals. The virus was detected in 28 out of 74 (37.8%) pigs tested by conventional PCR. Out of these 28 positive cases, 26 piglets were pre-weaned piglets (13 stillborn and 2 mummified fetuses). Three positive cases were used for complete genome sequencing. The complete genome of PCV2 in three overlapping fragments of size 661 bp, 767 bp and 676 bp of 3 isolates (PCV2-IZN-2016, PCV2-IZN-2015, IZN-572-2016) were subjected to direct sequencing. The combined sequences were submitted in GenBank with following accession numbers MK035433 (IZN-572-2016), MK035432 (PCV2-IZN-2016), MK035434 (PCV2-IZN-2015)

Genetic diversity and phylogenetic analysis of porcine circovirus 2: BLAST analysis of full genome of PCV2-IZN-2016 and PCV2-IZN-2015 revealed 99% homology with different isolates from China, Vietnam and recombinant strains (PCV2Izn-218-13 and PCV2Izn-89-13) from India. The sequences obtained from this study were analyzed along with other sequences retrieved from NCBI database to construct a phylogenetic tree. The isolate PCV2-IZN-2016 and PCV2-IZN-2015 clustered with different recombinant strains from India (PCV2Izn-218-13 and PCV2Izn-89-13) and China (HQ395058 and HQ395049). Phylogenetic analysis of IZN-572-2016 revealed that it belongs to genotype PCV2b-1A/B (Fig. 1). IZN-572-2016 exhibited high nucleotide identity with isolates from China, India, Lithuania and Switzerland.

Earlier studies from India suggesting genotypes circulating in India are PCV2a–2D, PCV2b–1C, PCV2d and recombinant strains. Further detailed analysis suggested that these strains evolved from inter-genotypic recombination between PCV2a–2C and PCV2b–1C genotypes within *cap* gene (Anoopraj *et al.* 2015). Since India shares boundary with Myanmar in the North eastern region and movement of pigs across the border is frequent, it is possible that genotypes sharing close homology with Chinese isolates gained access into the Indian pig population through this route. Another source may be pigs imported from European countries as a part of breeding programme to upgrade the native breeds which results in exchange of diseases between countries.

In PCV2 genome, high degree (around 93%) of nucleotide conservation was noticed worldwide. Despite being a DNA virus, it shows substantial genetic variations and evolutionary dynamics similar to RNA viruses (Segales *et al.* 2013). PCV2 genetic diversity is mainly due to recombination and mutation happening in the genome resulting in evolution of new types (Csagola *et al.* 2006). Recent studies on PCV2 epidemiology have focused on the emergence of new variants and recombinant strains of PCV2. Point mutation together with recombination leads to evolution of new clusters of PCV2 (Franzo and Segales 2018, Ramos *et al.* 2013). The isolate PCV2-IZN-2015 and PCV2-IZN-2016 were integrated into the recombinant cluster that provided proof for possible recombination event and these viruses shared high nucleotide identity with recently reported clusters of recombinant strains from India and China (Cai *et al.* 2012, Anoopraj *et al.* 2015).

The isolates from Northeastern region of India also used in analysis suggesting clustering of northeastern isolates into PCV 2a-2D, PCV 2b-1C, PCV 2b-1A/B, PCV 2d-2 and two new cluster. The comparison of ORF-2 gene among isolates from this region revealed a nucleotide identity ranging between 90.3% to 100% indicating the genetic diversity of PCV2 strains circulating in pigs of Northwestern region of UP. Latest reports in the north east region of India suggests the circulation of PCV2d and a new cluster similar to current study (Mukherjee *et al.* 2019, Barman *et al.* 2017)

The nucleotides with accession numbers LC004754.1 (Sikkim, SK-1), LC008140.1 (Meghalaya, ML-11), LC008139.1 (Assam, AS-3), LC004741.1 (Meghalaya, ML-6), LC004737.1 (Meghalaya, ML-2), LC004740.1 (Meghalaya, ML-5), LC004733.1 (Arunachal Pradesh, AR-2), LC008142.1 (Tripura, TR-1), LC004742.1 (Meghalaya, ML-7), KX009482.1 (Mizoram, PCV2/MZ/IND/43, LC008136.1 (Assam, AS-1), LC004744.1 (Meghalaya, ML-9), LC004743.1 (Meghalaya, ML-8) clustered separately belongs to 2d genotype and KJ729075.1 clustered with various isolates from China with 2d-2 genotype (HM038031.1, KC515005.1, HM038030.1, HM038017.1). In a study by Barman *et al.* (2017) stating the circulation of PCV2d genotype in Meghalaya and Assam.

The isolates MN-1 (Manipur, LC004735.1), India-GN-

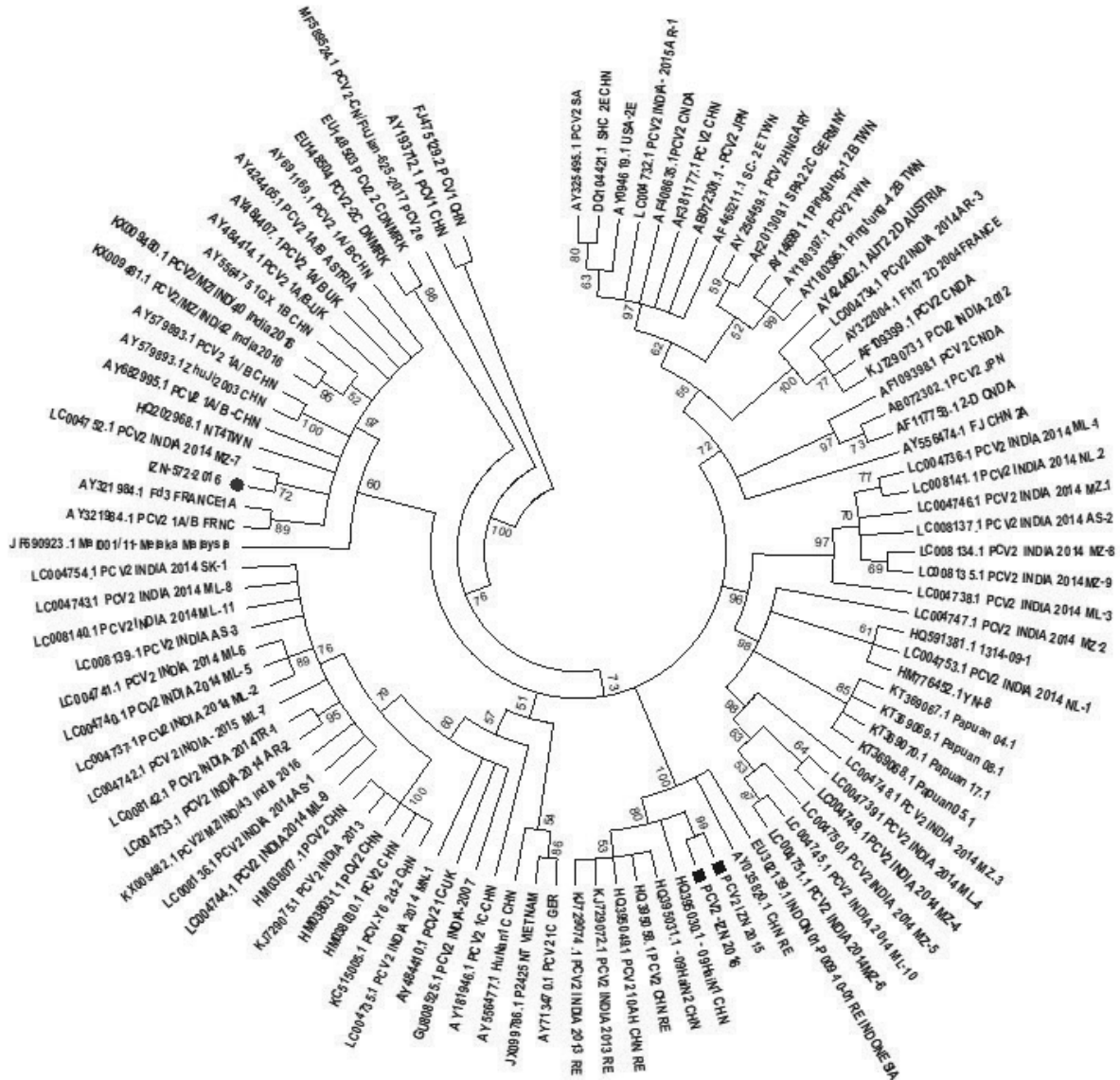


Fig. 1. Phylogenetic tree of PCV2 full genome using maximum likelihood method.

07 (Uttar Pradesh, GU808525.1) belongs to genotype PCV2b–1C. It clustered along with other 2b isolates from China, Vietnam and Germany. Isolate IZN-572-2016 (Uttar Pradesh), MZ-7 (Mizoram, LC004752.1), PCV2/MZ/IND/42 (Mizoram, X009481.1), PCV2/MZ/IND/40 (Mizoram, KX009480.1) belongs to PCV2b–1A/B clustered along with isolates from Malaysia, China, Uttar Pradesh and Austria. In PCV2b–1A/B group falls the isolates from Mizoram and Uttar Pradesh.

The isolates AR-3 (Arunachal Pradesh, LC004734.1), PCV2Izn-200-12 (India, KJ729073.1), AR-1 (Arunachal Pradesh, LC004732.1) belongs PCV2a genotype. The isolates ML-1 (Meghalaya, LC004736.1), NL-2 (Nagaland, LC008141.1), MZ-1 (Mizoram, LC004746.1), AS-2 (Assam, LC008137.1), MZ-8 (Mizoram, LC008134.1), MZ-9 (Mizoram, LC008135.1) and ML-3 (Meghalaya, LC004738.1), forms a separate cluster not belonging to

anyone of the existing genotypes. Also isolates NL-1 (Nagaland, LC004753.1), MZ-5 (Mizoram, LC004750.1), MZ-3 (Mizoram, LC004748.1), ML-4 (Meghalaya, LC004739.1), MZ-4 (Mizoram, LC004749.1), ML-10 (Meghalaya, LC004745.1) and MZ-6 (Mizoram, LC004751.1) clustered with the unclassified isolates from Croatia (HQ591381) and Indonesia (KT369067, KT369070, KT369068). Similar findings in a study conducted by Mukherjee *et al.* (2019) in these Meghalaya isolates. The study indicates the novel cluster was originated by inter-genotypic recombination between PCV2c and PCV2d.

Phylogenetic analysis based on the ORF2 (cap gene) is suitable for typing of PCV2 isolates into 4 different genotypes (Fig. 2). For studying the evolution of viruses, phylogenetic analysis is a potent tool. Mutation and recombination mainly reported from the ORF2 region of

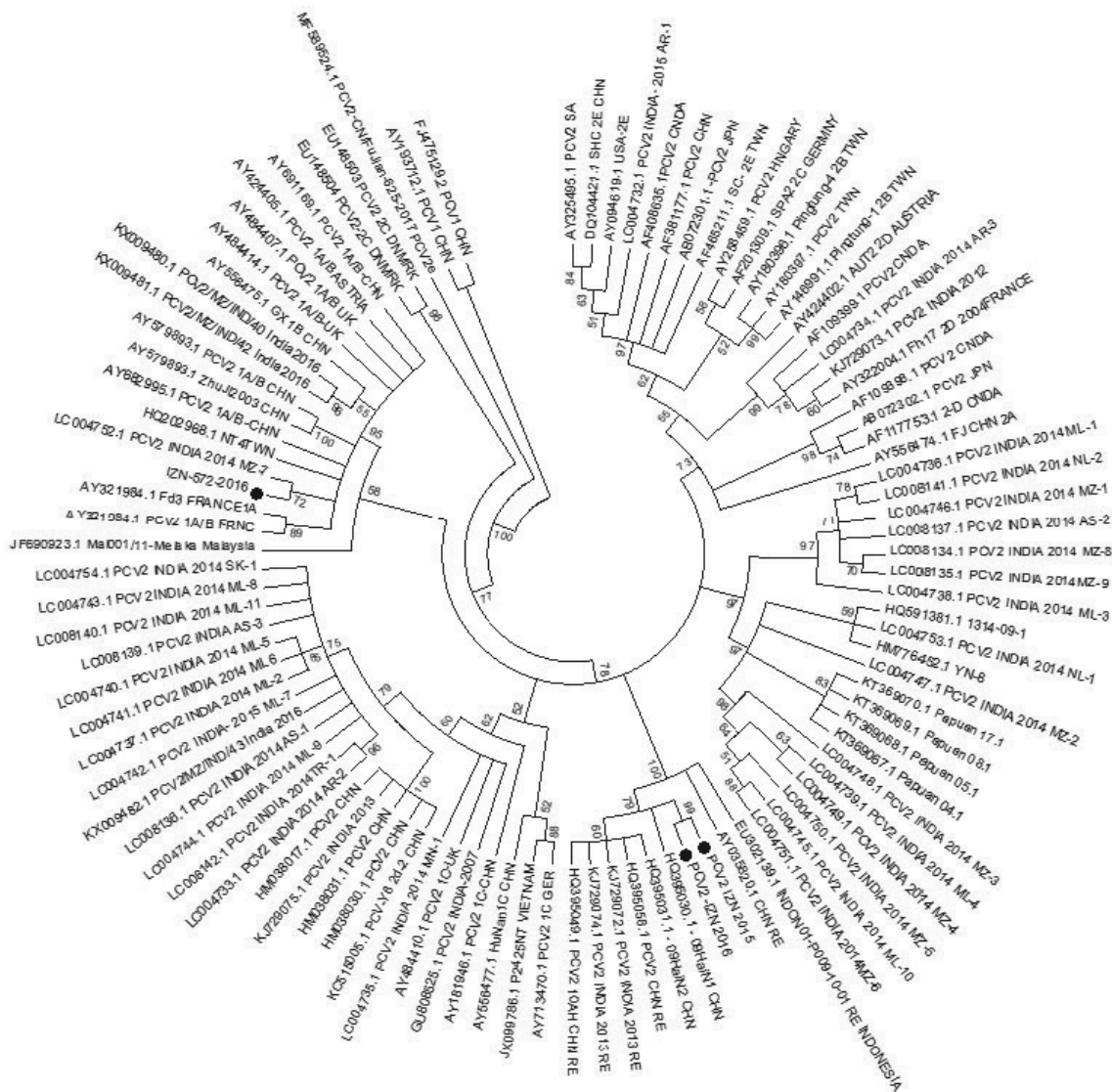


Fig.2. Phylogenetic tree of PCV2 ORF2 gene using maximum likelihood method.

PCV2, so this region can be selected for the phylogenetic analysis along with the full genome analysis (Franzo *et al.* 2016). The classification of isolates into clusters within the genotypes is better achieved by phylogenetic analysis using entire viral genome. Genotypic analysis using complete genome is essential to elucidate the genetic variation in terms of recombination, in which ORF1 is the frequent site (Ramos *et al.* 2013). In our study, analysis based on *cap* gene and complete genome resulted in similar phylogenetic trees.

Co-infection of animals with different genotypes or viruses of different clusters of same genotype results in recombination to occur (Khaiseb *et al.* 2011). PCV2 has been recorded from India since 2006 (Sharma and Saikumar 2008). Additionally, many genotypes and different clusters of same genotypes are circulating among Indian pigs. Therefore, it is highly likely that the natural inter-genotypic recombination has happened within this closed herd over a

period of time. PCV2 2a genotype is the most prevalent worldwide until 2003, due to genetic shift later prevalence changed to PCV2 2b. A similar situation have occurred since the appearance of the genotype 2d in 2010 which is rapidly spreading with detriment to PCV2b prevalence (Guo *et al.* 2010, Xiao *et al.* 2015)

In conclusion, in the present study, an investigation was carried out to identify the genetic differences of PCV2 isolates prevailing in different part of India and this study supplements knowledge on PCV2 genetic diversity in India. Phylogenetic analysis of 40 Indian PCV2 genomes downloaded from GenBank along with three new isolates from the current study based on the complete genome and *cap* gene together with nucleotide sequences of PCV2 isolates from different countries resulted in a tree in which Indian isolates clustered in 4 different branches namely PCV2b-1C, PCV2a-2D, PCV 2b-1A/B, PCV 2d-2 recombinant group and two new cluster in which one

clustered along with unclassified PCV2 viruses from Indonesia and Croatia. The comparison of ORF2 gene among Indian isolates revealed nucleotide identity ranging from 88.6% to 99.6%, indicating the genetic diversity of PCV2 strains circulating in Indian pig. The present work reports for the first time in India the PCV2-1A/B cluster of 2b genotype and all the Indian isolates available in India from 2006–2018 were used in this analysis.

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