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Molecular characterization of porcine circovirus 2 circulating in Assam and Arunachal Pradesh of India

Ajai Lawrence D'silva[a]*, Arpita Bharali[b]*, Lukumoni Buragohain[b]*, Dinesh C. Pathak[a], Narayan Ramamurthy[a], Rahul Batheja[a], Asok Kumar Mariappan[c], Sophia M. Gogoi[d], Nagendra Nath Barman[d], Sohini Dey[a], and Madhan Mohan Chellappa[a]

[a]Recombinant DNA Laboratory, Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Bareilly, India; [b]Department of Animal Biotechnology, College of Veterinary Science, Assam Agricultural University, Guwahati, India; [c]Division of Veterinary Pathology, Indian Veterinary Research Institute, Bareilly, India; [d]Department of Veterinary Microbiology, College of Veterinary Science, Assam Agricultural University, Guwahati, India

ABSTRACT

PCV2 is the primary etiological agent of porcine circovirus-associated diseases (PCVADs) which affect pigs worldwide. Currently, there is a worldwide genotype prevalence switch from PCV2b to PCV2d, which has led to increased virulence of the circulating virus strains leading to vaccine failures and selection pressure. In the present study, the PCV2 genotypes circulating in north eastern region (NER) of India particularly the states of Assam and Arunachal Pradesh was characterized by isolation, sequencing and phylogenetic analysis of *cap* gene. The phylogenetic analysis revealed that the PCV2 isolates circulating in pigs of Assam and Arunachal Pradesh were mostly of PCV2d genotype. Hence, it can be concluded that PCV2d genotype is the most dominating genotype in NER and priority should be given to this genotype for development of future vaccine candidate against PCV2 in India.

KEYWORDS

Cap gene; North-East India; PCV2d genotype; phylogenetic analysis

Introduction

Porcine circovirus 2 (PCV2) is a circular single-stranded DNA virus belonging to the family *Circoviridae* and genus *Circovirus*.[1–3] Currently, four types of PCV are reported which are designated as PCV1, PCV2, PCV3 and PCV4.[4] PCV2 is a major infectious agent involved in post weaning multisystemic wasting syndrome (PMWS)[5,6] and is an emerging and multifactorial disease with worldwide prevalence.[7] Initially, PCV2 was classified into PCV2a and PCV2b based on nucleotide diversity of capsid protein-encoding *cap* gene or ORF2.[8,9] Subsequently, genotypes PCV2c were reported from pigs of Denmark, Brazil and China.[10–12] PCV2d was detected in Switzerland, but later it spread rapidly with higher virulence in US swine herds.[13] Earlier it was called a mutant of PCV-2b (mPCV-2b) and various vaccination failure cases were reported in US[14] with PCV2d now prevalent worldwide. Two other genotypes, PCV2e was identified in US and Mexico[15] and PCV2f was reported from China.[16] Recently, two more genotypes (2 g and 2 h) of PCV2 were reported based on intra-genotype p-distance and bootstrap value.[17] The genome of PCV2 consists of 1766–1768 nucleotides with two major ORFs; ORF1 coding for replicase (*rep*) protein of 35.7 kDa and ORF2 coding for capsid (*cap*) protein of 27.8 kDa.[18] Additional two proteins are encoded by ORF3 and ORF4. Of this, the *cap* protein acts as the major immunogenic protein of the virus and provides complete protection against PCV 2 infection.[19]

PCV2 is detected in pigs of several states of India including the north-eastern region.[20–25] The most encountered genotypes in India were PCV2a, PCV2b and PCV2d[22–24] along with certain recombinant strains.[20,23,24] The earlier reports of PCV2 infection in Indian pig populations hint that PCV2 is likely to be endemic in India. Currently, there is no indigenous vaccine produced in India and therefore to generate an effective vaccine the genetic diversity of PCV2 circulating in India particularly in NER having high pig

CONTACT Sohini Dey sohinimadhan@gmail.com; Madhan Mohan Chellappa madhansohini@gmail.com Recombinant DNA Laboratory, Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Bareilly, India.
*These authors contributed equally for the work.

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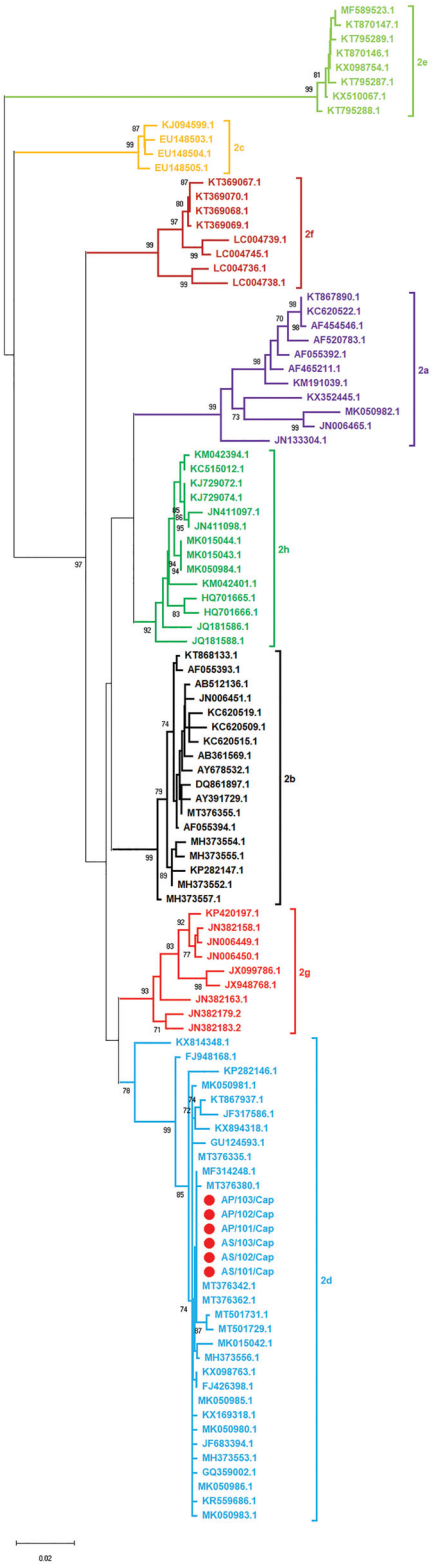

Figure 1. Phylogenetic tree of PCV2 based on cap gene sequences. The tree was constructed in MEGA X software by Neighbor-Joining (NJ) method. The eight PCV2 genotypes were represented in different color clades along with respective GenBank Accession numbers. The cap gene sequences of PCV2 used in this study formed a clade with genotype PCV2d which are highlighted with red solid circular marker.

population is a pre-requisite. The present study aims to identify the dominating PCV2 genotypes circulating in Assam and Arunachal Pradesh of NER.

Materials and methods

Virus samples and isolation

Six numbers of PCV2 positive tissue samples were obtained from repository of Advanced Animal Disease Diagnosis and Management Consortium (ADMaC) Laboratory, College of Veterinary Science, Khanapara, Guwahati, Assam, India. Three samples belonged to those collected from different parts of Assam and the other three were received from Arunachal Pradesh. All the samples were collected from suspected pigs having a clinical history of PCV2 infection and were found to be positive for PCV2 in initial PCR screening conducted at ADMaC Lab, however, these samples were not characterized earlier.

PCV2 was isolated in PK-15 cell line (PCV free) by the method as described previously with slight modifications.[26] Briefly, virus inoculum was prepared from pooled tissue samples. PK-15 cells grown in 25 cm^2 cell culture flask was inoculated with 1 ml of virus inoculum and incubated for 5 h at 37 °C in a 5% CO_2 incubator. After that, cells were treated with 1 ml of 300 mM D-Glucosamine and incubate for 30 minutes at 37 °C. Then, D-Glucosamine was discarded and cells were washed twice with plain M199 media. Following this, fresh media containing 4% FBS with antibiotic mixture was added and incubated for 72 h at 37 °C in a 5% CO_2 incubator. After 72 h of incubation, the virus particles were harvested by subjecting the cells to three cycles of freeze-thawing. The cells were centrifuged and supernatant was used as inoculum for subsequent four passages.

PCR amplification of PCV2 cap gene and sequencing

Total DNA was extracted from 4th passaged cells containing virus using DNeasy Blood and Tissue Kit (QIAGEN, Germany) as per manufacturer's instruction and presence of PCV2 was confirmed by amplifying ORF2 (*Cap* gene) of PCV2 using differential PCR primers.[27] PCR was carried out in a Thermocycler (Applied Biosystems, US) with conditions of 95 °C for 10 min as initial denaturation followed by 40 cycles of 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 1 min followed by final extension of 10 min at 72 °C. The PCR products were subjected to agarose gel electrophoresis followed by purification of the amplified products

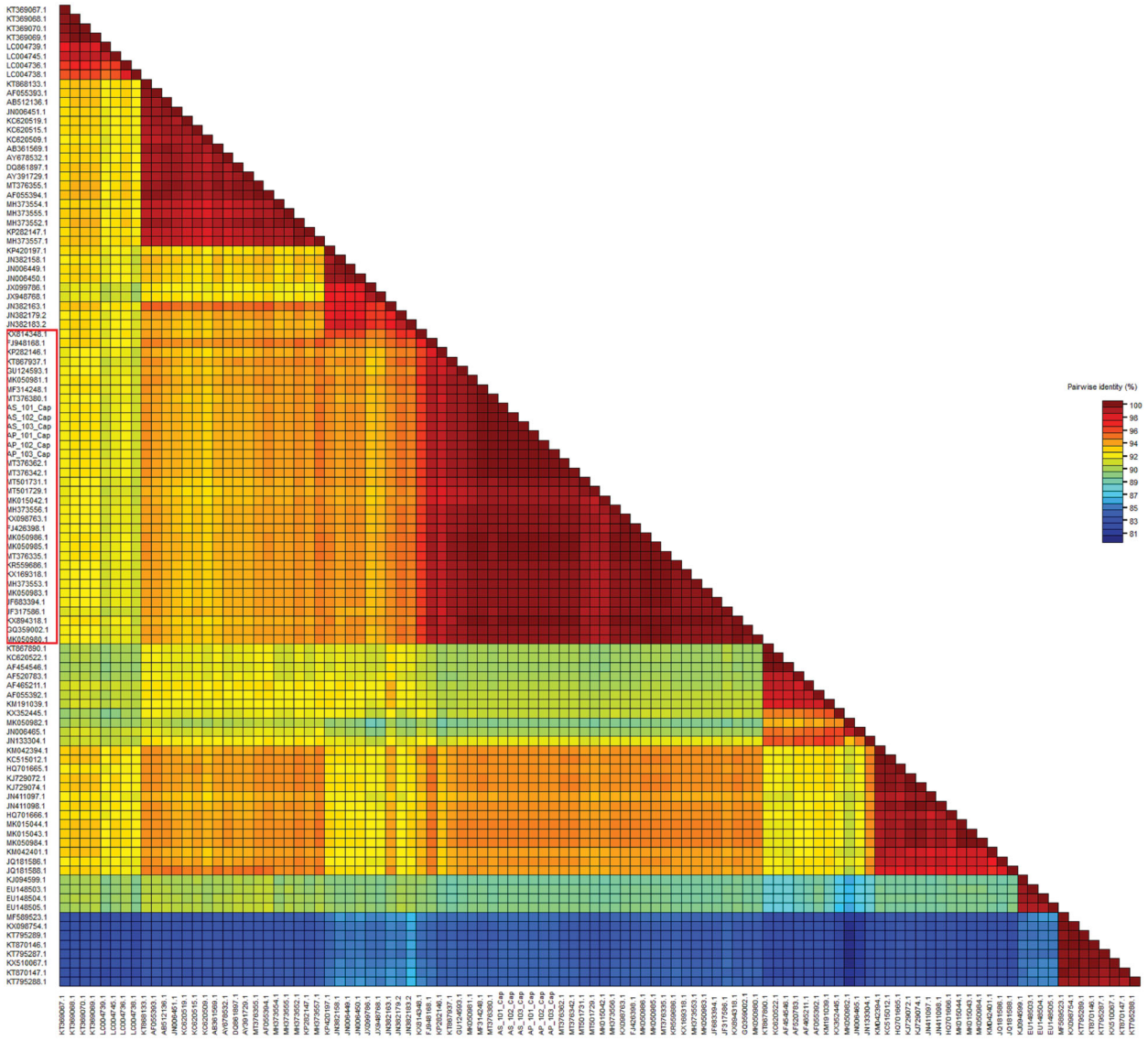

Figure 2. Pairwise identity matrix of cap gene sequences of PCV2 genotypes. Color-coded pairwise identity matrix of PCV2 cap gene generated by using SDT program. Each colored cell represents a percentage of identity score between two sequences (one indicated horizontally and other vertically). The PCV2d genotype forming a sub-molecular group is indicated by red box.

using QIAquick® gel extraction kit (Qiagen, Germany). The purified products were sequenced by Sanger sequencing.

Phylogenetic analysis

Capsid (*cap*) gene sequences (100 numbers) of PCV2 belonging to different genotypes (PCV2 a-h) as reported by Franzo and Segalés[17] as well as *cap* gene sequence of PCV2 detected in other species (Table S1) were retrieved from NCBI GenBank database (https://www.ncbi.nlm.nih.gov/). The phylogenetic analysis was done in MEGA X software[28] with 106 *cap* gene sequences including the sequences of the PCV2 isolates considered in this study. Alignment of multiple sequences was carried out using the CLUSTALW program implemented within MEGA X. All positions that contained alignment gaps and missing data were eliminated from the analysis. Phylogenetic tree was constructed using the Neighbor-Joining (NJ) method[29] based on Tamura-Nei substitution model[30] and statistical significance of the tree was tested by bootstrap analysis[31] of 1000 pseudo-replicates.

Pairwise sequence identity and distance of cap gene at nucleotide level

Sequence Demarcation Tool (SDT) v1.2[32] was used to estimate the pairwise identity. Based on phylogenetic and pairwise identity results, p-distances of the

isolated PCV2 genotypes were calculated in MEGAX[28] by comparing with other nucleotide *cap* gene sequences of the same PCV2 genotype and matrix of nucleotide divergence within the genotype was generated using 1000 bootstrap replicates to estimate variance.

Results and discussion

The fourth passaged viruses were confirmed by PCR amplification of the complete *cap* gene followed by DNA sequencing. All the viruses belonged to the PCV2d type by differential PCR. After analysis of the DNA sequences, the phylogenetic tree was constructed by NJ method using *cap* gene sequences of PCV2 depicting eight distinguished clades belonging to different genotypes (Fig. 1). In the phylogenetic analysis, it was elicited that all the PCV2 isolates of Assam and Arunachal Pradesh formed clade (red, highlighted with red solid circular marker) with PCV2d genotype, which reveals that the isolated PCV2 belongs to genotype PCV2d.

The pairwise sequence identity analysis done by using SDT tool represents that the identity of *cap* gene sequences of PCV2 belonging to eight different genotypes (a–h) considered in this study were between 80% and 100% (Fig. 2). Pairwise sequence identity analysis also revealed that PCV2 isolates formed submolecular group with PCV2d genotype (Fig. 2; highlighted in red box) which further confirmed the results of phylogenetic analysis. The overall nucleotide diversity of *cap* gene among PCV2d strains was found to be between 0 and 4% (Table S2). However, the *cap* gene of PCV2 isolates of Assam and Arunachal Pradesh have shown 0.00–3.4% divergence with other PCV2d strains and no diversity was found among the isolates of Assam and Arunachal Pradesh (Table S2).

Phylogenetic analysis and pairwise sequence identity revealed that the PCV2 isolated from domestic pig of Assam and Arunachal Pradesh belonged to genotype PCV2d. Thus, it indicates the circulation of PCV2d genotype in pig population of North-East India which is in consensus with earlier reports wherein it was already established the circulation of PCV2d genotypes in pig population in the north eastern states of India.[22,23,33] Although, previous studies reported the circulation of PCV2a, PCV2b and PCV2d genotypes along with certain recombinant strains,[20,22–24] but this study suggests that the genotype PCV2d is getting endemic within the pig population in the NER and there's an urgent need to control and eradicate the disease. Moreover, this suggests a shift of PCV2 genotypes to PCV2d within the NER of India which warrants more molecular and epidemiological studies in large representative samples to reflect on the trend and distribution of assorted strains within the country.[22]

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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