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Identification of a novel cluster of PCV2 isolates from Meghalaya, India indicates possible recombination along with changes in capsid protein

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ABSTRACT

Documentation of the emergence of Porcine circovirus 2 (PCV2) infection and economic losses incurred due to high mortality has been reported worldwide. The prevalence and genetic diversity of the virus has been reported in Northeast India including the possible chances of Classical swine fever virus (CSFV) vaccine failure in pig population in this region resulting in major disease outbreak. Irrespective of the genetic variability, the emergence of a novel cluster (based on the ORF2 phylogeny) was reported last year. The present study describes a state-wide (Meghalaya, India) molecular epidemiological investigation of PCV2 strains in pig population by amplification, sequencing and undertaking phylogenetic analyses. The results indicate the identification of a novel cluster of PCV2 originating from the inter-genotypic recombination between PCV2c and PCV2d. Multiple sequence alignment of amino acids indicates possible substitution in the A, B and C domains of the capsid protein. Molecular structural modelling of the capsid protein of PCV2 indicated possible motif variations in the secondary structure including presence of a tunnel, encountered at the interface region on each chain facilitating in transportation of molecules and acting as an active site for attachment and penetration. The baseline data strengthens the existing control programme of PCV2 and is possibly helpful in the planning of active surveillance strategy in this region.

1. Introduction

Porcine circovirus 2 (PCV2) is a member of *Circoviridae* family, genus *Circovirus*, it is a small, non-enveloped, icosahedral virus with a single-stranded 1767–1768 nucleotides (nt) long circular DNA genome (Hamel et al., 1998). PCV2 was first detected in Canada in 1997, followed by its reports from other parts of the world as a causative agent of post-weaning multisystemic wasting syndrome (PMWS) (Chae, 2005). Genome of PCV2 is composed of three major open reading frames [ORFs]. ORF1 encodes the replication associated protein (Rep); ORF2 encodes the capsid protein (Cp) and ORF3 encodes for an apoptotic protein (Liu et al., 2005) another recently described study suggests that ORF4 is embedded into ORF3 in the same direction and helps to promote PCV2 infection through suppressing caspase activity and $CD4^+$ and $CD8^+$ T cell (He et al., 2013). Cp is used as a representative phylogenetic marker and based on the ORF2 sequence distance, PCV2 is divided into three genotypes; PCV2a, PCV2b and PCV2c, genetic distance between these genotypes is 0.035 (Cortey et al., 2011). PCV2a is further divided into five clusters (2A-2E), PCV2b is divided into three clusters (1A, 1B, 1C), PCV2c has no subdivision, and the average distance within the cluster of PCV2a and PCV2b is 0.0158 and 0.0234, respectively (Grau-Roma et al., 2008). A variant of PCV2 strain, with an elongation of ORF2 by one amino acid (Lysine (K)) was identified in several PCV2 infection cases in United States (Xiao et al., 2012), is now classified as PCV2d nowadays. Recently a novel circovirus significantly divergent from PCV1 and PCV2, identified in US swine herds with approximately 2000 nt long genome classified as PCV3, has a potential association with multi-systemic disease and reproductive failure similar to PCV2 (Phan et al., 2016; Palinski et al., 2017).

Northeast India is comprised of eight states (Meghalaya, Assam, Manipur, Mizoram, Nagaland, Arunachal Pradesh, Tripura and Sikkim) and is a home to nearly 40% of the total pig population in India where pig husbandry plays an inseparable role in socio-economic status of this region (Bett et al., 2012). Livestock census report (2012) from Animal Husbandry Department of Meghalaya reported pigs make up 30% of total livestock (Livestock Census Report-2012, Government of India). Porcine viruses like CSFV, PCV2, Porcine reproductive and respiratory syndrome virus (PRRSV) are predominant in the state (Mukherjee et al.,

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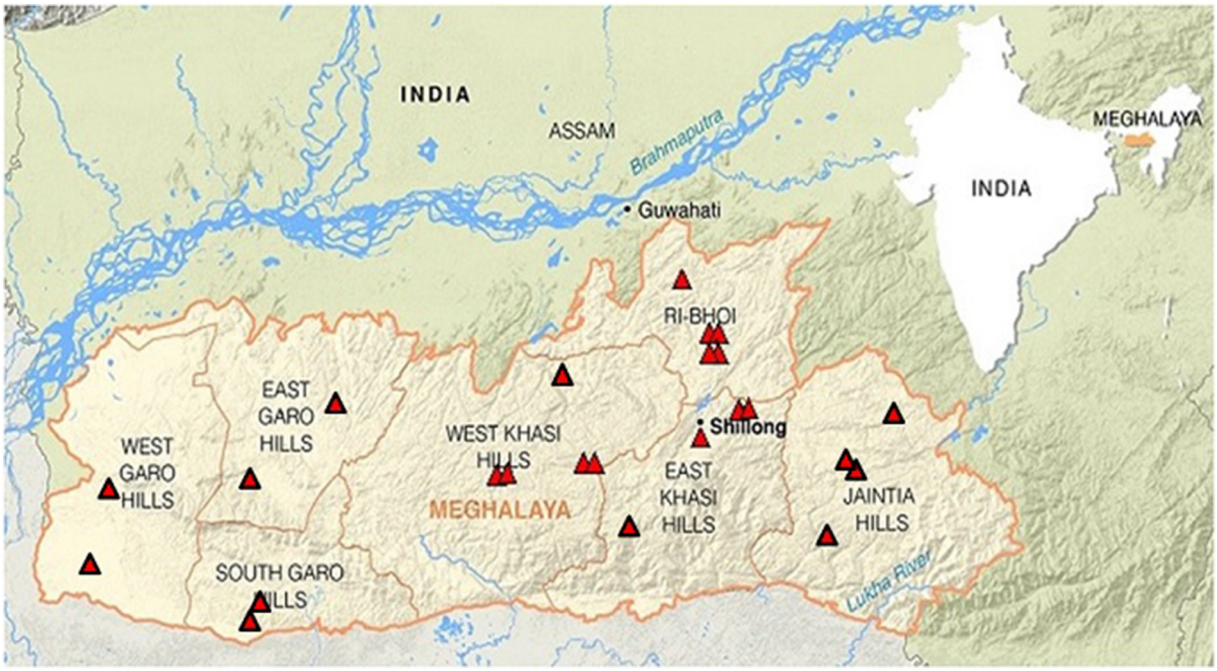

Fig. 1. Map of Meghalaya indicating the samples collection point during the period of December 2016 to June 2018.

Table 1
Oligonucleotide primer sequences for PCV2 detection and whole genome amplification.

Virus	Primer	Sequence(5′- 3′)	Target region (bp)	Reference
PCV2 (ORF2)	PCV2 F	CGG ATA TTG TAG TCC TCG TGG	1095–1115	Kim et al., 2001
	PCV2 R	ACT GTC AAG GCT ACC ACA GTC A	1570–1549	
PCV2 (Full)	PCV2 Full F	CCCCGTTGGAATGGTACTCCTCAACTG	823–849	Cai et al., 2012
	PCV2 Full R	CGGGGTCTGATTGCTGGTAATCAGAAT	827–810	

Table 2
Details of the characterized positive samples along with GenBank accession numbers.

Strain No.	Place of collection	Year of Collection	GenBank Acc.No.
T-800	Pynsursla, East Khasi hills	2016	MH496613
T-903	Mawryngkneng, Jaintia hills	2018	MH496614
T-935	Krydemkulai, Ri-Bhoi	2016	MH509731
T-936	Mairang, West khasi hills	2017	MH509732
T-943	Baghmara, South Garo hills	2016	MH509734
T-942	Betasing, South west Garo hills	2018	MH509735
T-945	Khliehtyrshi, Jaintia hills	2018	MH509736

2018a, 2018b) and frequent CSF outbreaks are documented in this region (Ahuja et al., 2015; Barman et al., 2003; Rajkhowa et al., 2014; Roychoudhury et al., 2014). Meghalaya is a landlocked state, bounded on the North by Assam, at the South and the South-west by Bangladesh. The genetic diversity of PCV2 based on the ORF2 gene sequences indicates the emergence of PCV2d strains in Meghalaya with possible natural inter-genotypic recombination event between PCV2a and PCV2b highlights the influence of PCV2 on the health and economy of pig husbandry in the region (Mukherjee et al., 2018b). Thus, the present study was envisaged to further document the molecular epidemiological status of PCV2 in Meghalaya. The study was undertaken to preliminarily assess the genetic identity of PCV2 strains circulating in the region along with diversity encountered if any based on whole genome and amino acid sequences.

2. Material and methods

2.1. Ethical approval

As per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines, study involving clinical samples does not require approval of Institute Animal Ethics Committee. However the samples collected for the present study followed standard sample collection methods without causing any harm or stress to the animals.

2.2. Sample collection

A total of 289 serum samples from grower pigs were collected from organised pig farms of Meghalaya with previous and frequent history of reproductive and respiratory failures, by the Division of Animal Health, ICAR and the District Investigation Office (DIO), for confirmatory diagnosis of the PCV2 infection. The period of sample collection was from December 2016 to June 2018 at bimonthly interval from the 3 geospatial locations of Meghalaya constituting the three hills division of the region (Fig. 1). Samples were brought to the laboratory under cold chain for the detection of PCV2 viral antibody, antigen and molecular characterization of the virus.

2.3. Antibody detection, nucleic acid extraction and PCR

Samples were screened by PCV2 specific antibody ELISA to obtain a steady and reliable assessment of antibody titers by using Ingezim Circo IgG.1.1.PCV.K.1 (INGENASA, Madrid, Spain) kit as per the manufacturer's protocol. The kit used for the antibody

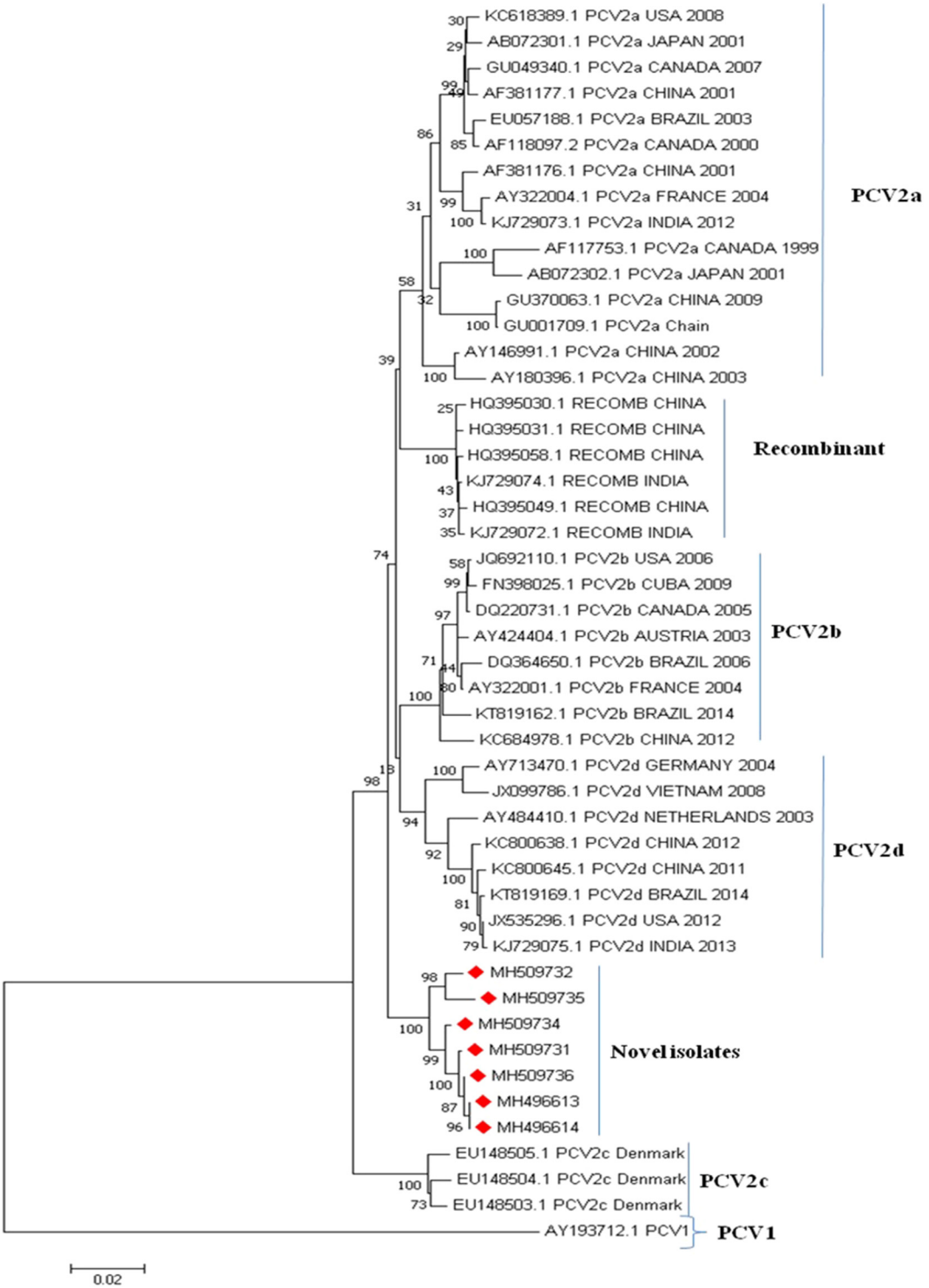

Fig. 2. Neighbour-Joining evolutionary tree constructed by *p*-distance model based on 47 PCV2 full sequences from the GenBank which comprised of 7 PCV2 isolates of the present study and 40 reference sequences. Sequence with Red dots represents isolates from the present study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detection of PCV2 was OIE recommended. All the samples were initially screened by antibody based ELISA kit and subsequently the positive serum samples were used for DNA extraction.

To ascertain the presence of circulating antibody bound complexes of the viruses in subclinical infections, nucleic acids from the serum samples were extracted. DNA was extracted from all ELISA positive sera samples, by using QiaAmp Viral DNA mini kit (Qiagen, Germany) according to manufacturer's instruction.

For the genomic detection of PCV2, ORF2 region of the virus was targeted which encodes the viral structural and virulence-associated proteins. A set of previously reported primers specific for partial ORF2 fragment was used for the detection of PCV2 and the complete genome of PCV2 was amplified by using the primer set (PCV2 Full) as described by Cai et al., 2012 (Table 1) in a thermal cycler [TaKaRa, Japan]. The PCR products were analyzed by electrophoresis in 1% agarose followed by ethidium bromide staining and visualization under ultraviolet light.

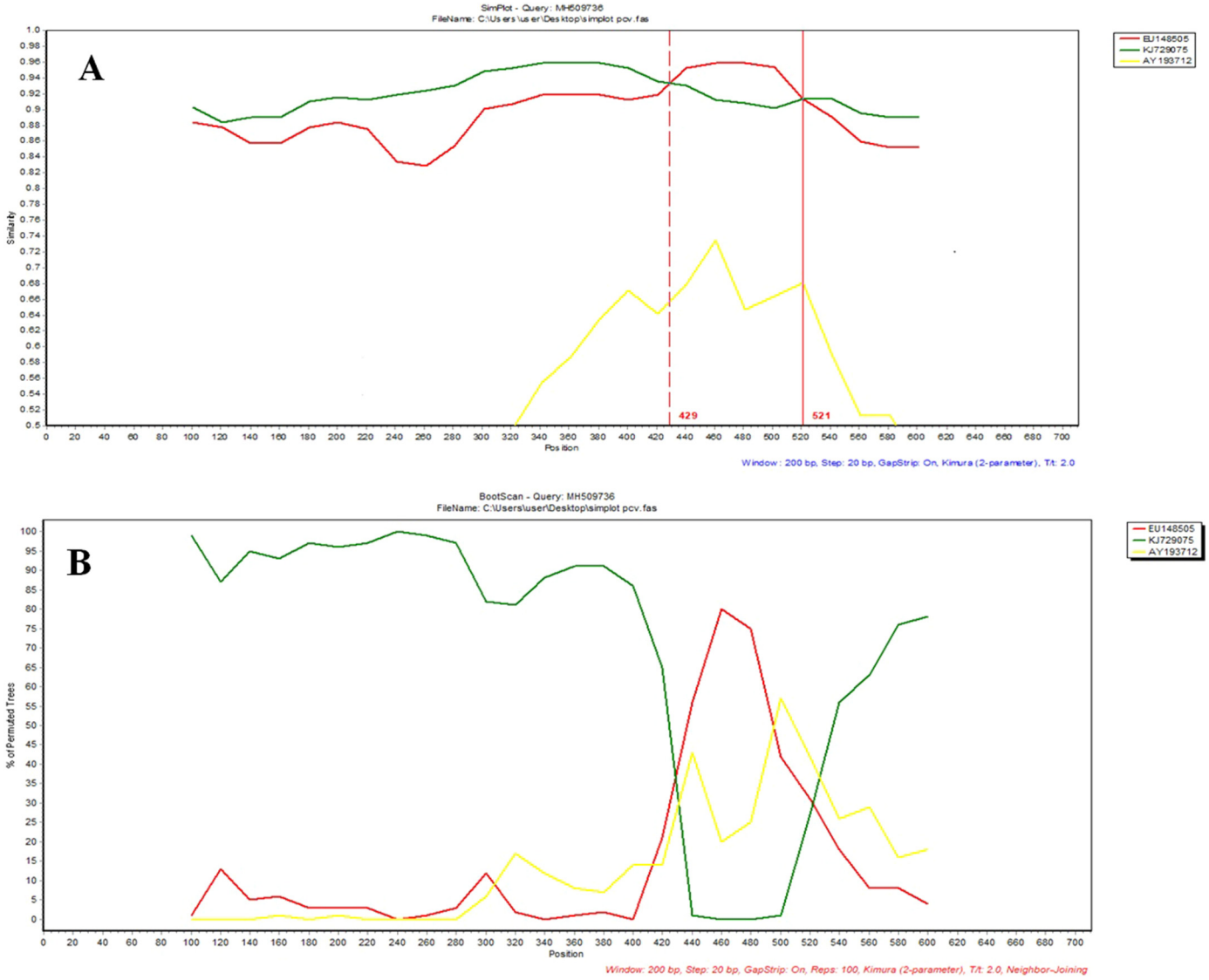

Fig. 3. Simplot analysis. (A) Similarity plot (B) Bootscan analysis generated with MH509736 as query sequence, KJ729075 and EU148505 as parent groups and AY193712 as PCV1 out-group with sliding window of 200 bp and step size of 20 bp; Kimura 2-parameter (100 Reps) was used as a distance model with transition-transversion ratio of 2. The Y-axis represents the % of permuted trees and the X-axis represents the position.

2.4. Sequencing and sequence analysis

All amplified PCR products (PCV2 Full) were purified from agarose gel using GeneJet gel extraction kit (Thermo Scientific, Lithuania, Europe] and ligated into vector pTZ57R/T vector (Thermo Scientific, Lithuania, Europe) with T4 DNA Ligase [Thermo Scientific, Germany] following TA cloning mechanism. The ligated products were used for the transformation and propogation in *E. coli* Top 10 competent cell [invitrogen, USA]. Recombinant clones among the transformed cells were screened by blue-white screening mechanism. White colonies were selected and inoculated into Luria Bertani [LB] [HiMedia, India] broth made selective with ampicillin and grown overnight at 37 °C in a shaker incubator. The plasmid DNA extraction was carried out by using GeneJET Plasmid Miniprep Kit [catalogue: K0503] [Thermo Scientific, Germany] following the manufacturer protocol. Purified recombinant plasmid clones were subjected to bidirectional nucleotide sequencing using BigDye in ABI 3500xL Genetic Analyzer automatic sequencer (Applied Biosystems). Raw sequences were assembled by SeqMan 6.00 (Lasergene 6 software package) software and sequence homology was searched by blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Complete sequences of the PCV2 Full gene were submitted to GenBank and the accession numbers accorded were indicated in Table 2.

2.5. Bioinformatics analysis

Multiple sequence alignment was carried out using ClustalW programme (Larkin et al., 2007) of MEGA v.7.0.21 software. Neighbour joining evolutionary tree was constructed by distance model (Kimura, 1980) as nucleotide substitution model based on the PCV2 Full sequences. A PCV1 isolate (AY193712) was used as an out group and the phylogenetic tree was rooted by this sequence. Test of phylogeny was done by bootstrap method and reliability of the constructed tree was determined by 1000 bootstrap replicates. Blast search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to find putative parents for the determination of recombinant event using putative recombinants (of this study) as queries. A pairwise distance comparsion was performed for nucleotides and amino acids using MEGA7. Recombination analysis was done using software SimPlot program (version 3.5.1) to calculate nucleotide identity as described by Lole et al. (1999).

Phylogenetic analysis was carried out by the construction of Neighbour-joining evolutionary tree based on the 47 PCV2 full sequences comprising of 7 PCV2 sequences of the present study (Table 2)

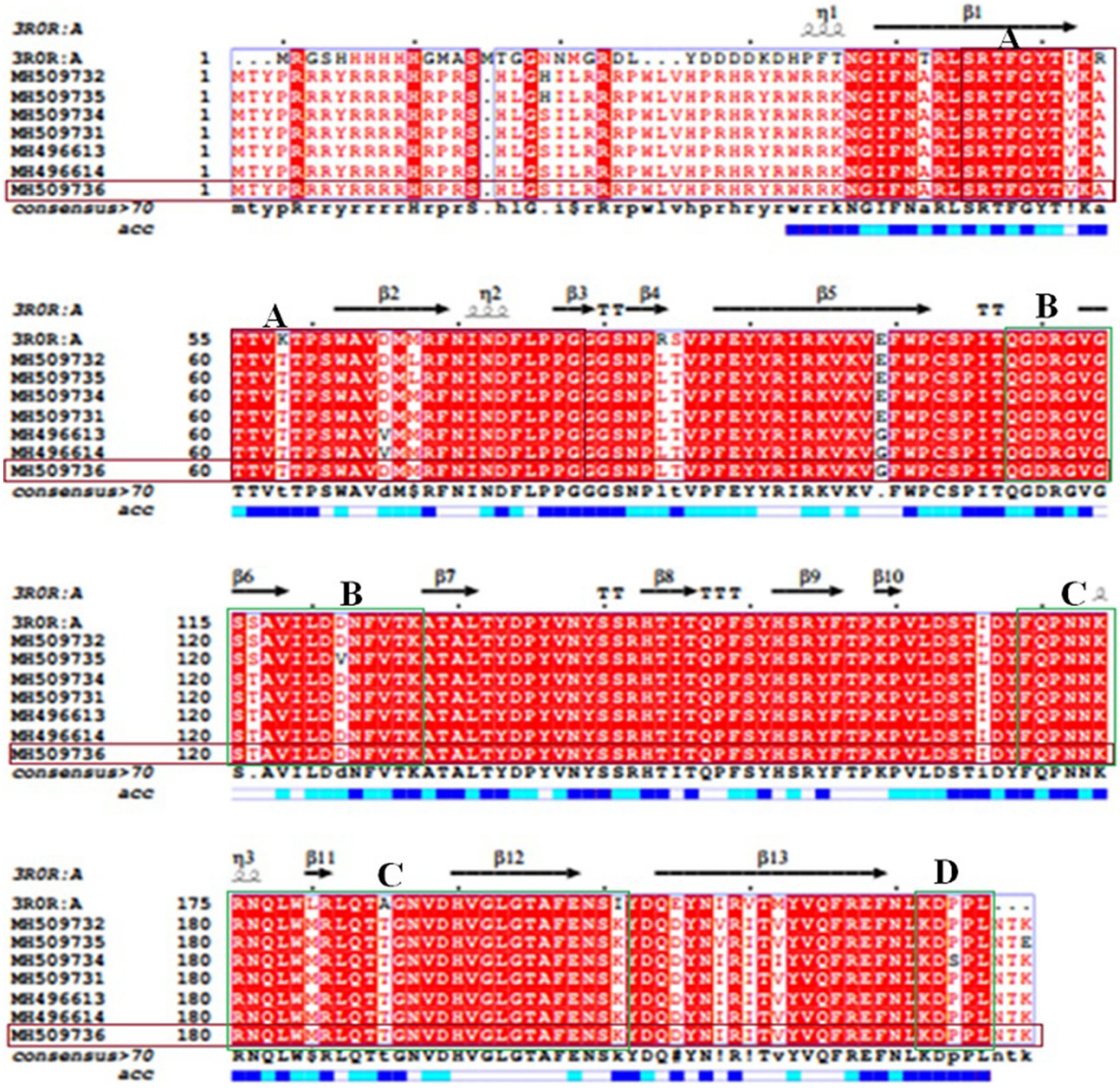

Fig. 4. Amino acid alignment of the capsid protein deduced in ESPript 3.0 software taking PCV2 consensus sequence (PCV^{CS}) (PDB ID: 3R0R: A) as putative parent. Sequence MH509736 (Highlighted) was taken as representative sequence of all the isolates. The highlighted areas (A, B, C, D) symbolises different antibody recognition domains of the capsid protein.

and 40 reference sequences from the GenBank. A PCV1 isolate (AY193712) was used as an out group and the phylogenetic tree was rooted by this sequence. Amino acid sequence of the capsid protein were aligned to the PCV2 consensus sequence (PCV^{CS}) (PDB ID: 3R0R:A) using the Clustal program (Chenna et al., 2003) and plotted by EPSprint server (Robert et al., 2014).

2.6. Molecular modelling of PCV2 capsid protein

A PCV2 sequence (MH509736) was selected for the generation of a representative model of the capsid protein of PCV2 using I-TASSER online server for protein structure and function prediction (http://zhanglab.ccmb.med.umich.edu/I-TASSER) (Roy et al., 2010; Yang et al., 2015; Zhang, 2008). The first step in I-TASSER includes the search of a structural template by LOMETS (meta-threading method containing eight-fold recognition programs) through a non-redundant structure library. The model of the representative sequence of this study was built using 3R0R:A structure (PCV2 monomer consensus sequence) as template, having the highest resolution required for the effective atomic position accuracy. The stereochemical quality and accuracy of the generated model was evaluated using Ramachandran plot analysis. The pentamer was generated by SYMMDOCK server (Schneidman-Duhovny et al., 2005) and the generated model was visualized using software RasMol program (version 2.7.5.2). Furthermore the generated model was submitted to PDBSum server (Laskowski, 2001) and ProFunc server (Laskowski et al., 2005) for the structural inference and

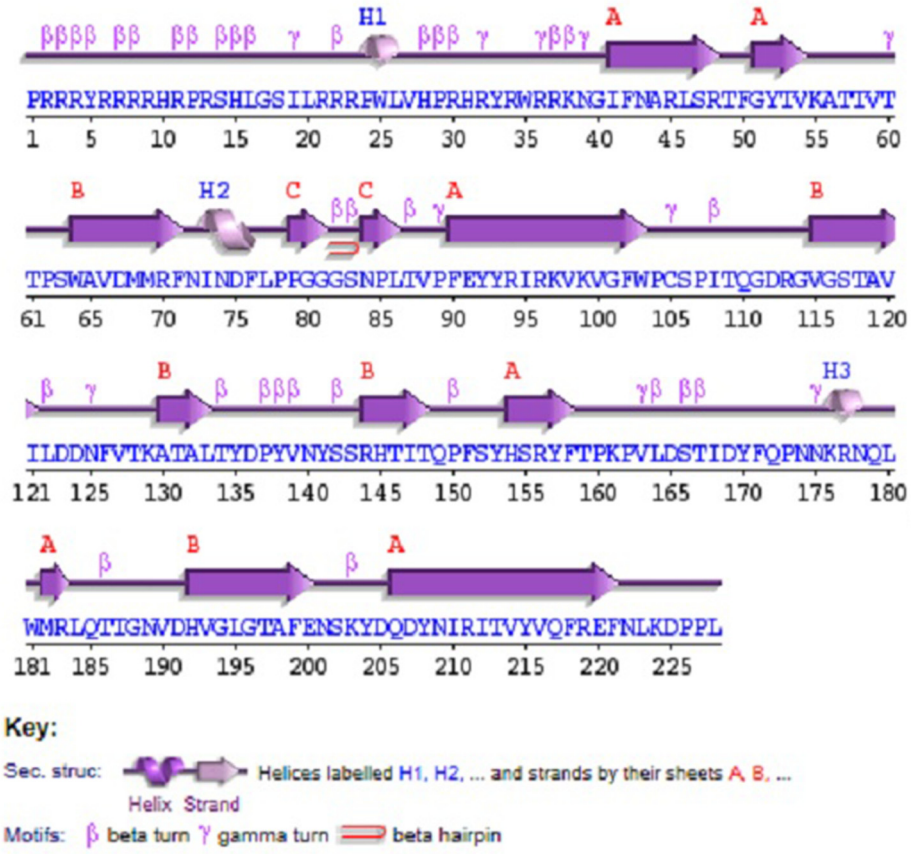

Fig. 5. Secondary structure- wiring diagram of the representative isolate MH509736 as generated by PDBsum online server. The motif of the capsid protein was described with colour and structure codings.

prediction of protein function.

3. Results

3.1. Seroprevalence and PCR detection of PCV2

Out of 289 serum samples screened, 191 samples were positive for PCV2 specific antibody giving a mean positivity of 66.09%. Among the 191 seropositive samples, a total of 59 samples were found positive by the detection primers of PCV2 based on the ORF2 region indicating an overall percentage value of 30.89%. All these 59 positive samples were amplified for PCV2 whole genome, out of which 7 representative samples from different districts of Meghalaya were sequenced, annotated and submitted in GenBank (Table 2).

3.2. Phylogenetic analysis

The complete genome sequence of all the PCV2 sequences had 1760–1767 nucleotides having two cds (CoDing Sequence) - Replication protein (314 amino acids) and Capsid protein (233–234 amino acids). Phylogenetic analysis of these sequences was carried out by the construction of Neighbour-joining evolutionary tree. The sequences of this study showed highest sequence identity of 97% to 99% with KJ729072 and HQ395058 sequences from India and China at nucleotide and amino acid level respectively. Phylogenetic analysis revealed that all PCV2 sequences of Meghalaya formed a separate cluster between PCV2c and PCV2d genotypes with reliable bootstrap values and were distinctly separated from other genotypes of PCV2 (Fig. 2), indicating a possible divergence.

3.3. Characterization of novel PCV2 recombinant cluster

Recombination event in the sequences were further characterized through similarity plot analysis and Bootscan analysis using Simplot software (Fig. 3). A representative PCV2 sequence (MH509736) was selected among them as query sequence. The crossover location in the *cp* gene indicated potent recombination of the query sequence with the putative parents KJ729075 (green), EU148505 (red) and out-group AY193712 (yellow).

The deduced amino acid for the capsid gene sequences of PCV2 from this study indicated highest identity with PCV2 consensus sequence (PCV^{CS}) (PDB ID: 3R0R:A) (Fig. 4). Multiple sequence alignment of the amino acid sequences represented the conserved and variable residues of PCV2 capsid protein of sequences from this study and the consensus sequence of crystallographic structure of 3R0R:A.

The secondary structure- wiring diagram of the representative sequence from this study was generated by PDBsum online server (Fig. 5). The motif of the capsid protein indicates 3 sheets, 1 beta hairpin, 5 beta bulges, 13 strands, 2 helices, 34 beta turns and 8 gamma turn. The motif of the representative sequence was compared with that of PCV2 consensus sequence (PCV^{CS}) (PDB ID: 3R0R: A) to determine the stability and helical characteristics of the capsid protein. The amino acid residues forming 2 helices in the capsid protein are polar amino acid type.

Model of capsid protein monomer of representative PCV2 sequence-MH509736 was generated by I-TASSER, based on the PCV^{CS} (PDB ID: 3R0R:A) and other structure determined by Cryo-EM or homology modelling as template. The deduced Ramachandran plot shows 98% residues in the allowed region, indicating the stability of the protein (Fig. 6). The Pentamer of the amino acid residues was generated by SYMMDOCK server and the generated model were visualized and compared with consensus capsid protein 3R0R by RasMol program. The

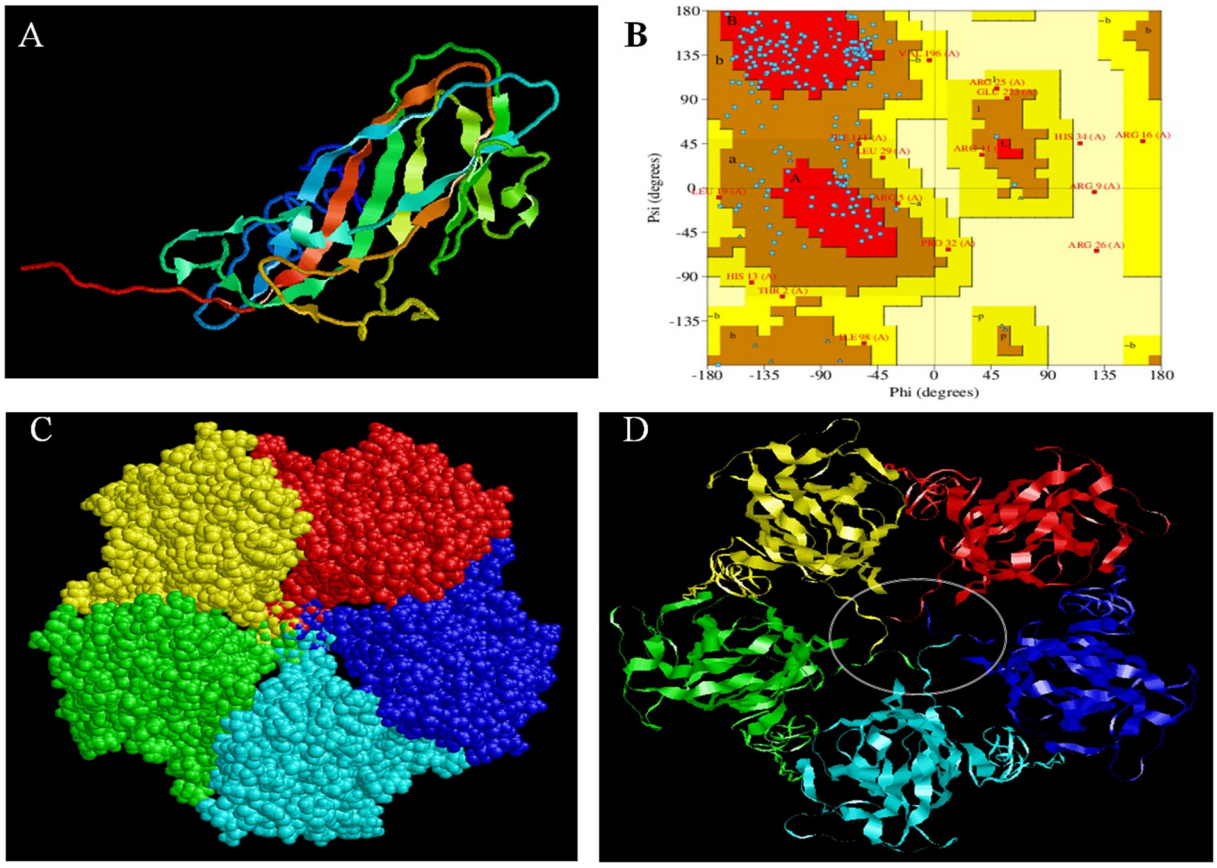

Fig. 6. Protein Modelling: (A) The 3D structures of MH509736 generated by I-TASSER online server. The best model was selected on the basis of highest C-score given by I-TASSER. (B) The Ramachandran plot shows 98% residues in the allowed region (C) Pentameric structure of MH509736 generated by SYMMDOCK online server represented in different colour group-wise (D) Capsid structure built using icosahedral symmetry by Chimera software.

Pentameric structure and the Capsid structure built using icosahedral symmetry by Chimera software indicated clefts of various volumes and specifically a tunnel, which indicates the presence of interior space in the capsid protein that connects with the protein subunit which is absent in the consensus. The tunnel is the Capsid structure built using icosahedral symmetry.

4. Discussion

Infection of PCV2 in a farm often represents subclinical infection (Ladekjær-Mikkelsen et al.; 2002) in which pigs may act as potential virus shedders (Brunborg et al.; 2004). Thus, regular observation and supervision of PCV2 infection is necessary through serology or molecular detection. Although PCV2 is considered as a principal causative agent of PMWS, several co-factors like bacterial and viral infection leads to enhancing the severity of the infection (Chae, 2005).

Evidence of PCV2 infection and its combined infection with CSFV and PRRSV in pig population has been reported from this region (Mukherjee et al., 2018a, b). The genetic variability of the PCV2 strains in this region has also been identified based on the ORF2 sequence (Mukherjee et al., 2018b). Thus, the influence of PCV2 on the health and economy of pig husbandry in Meghalaya has been investigated. The present study focuses on the identification and characterization of a novel cluster of PCV2 based on whole genome sequencing.

The serum samples collected from December 2016 to June 2018 from different regions of Meghalaya indicated a mean positivity of 60.09% (191/289) and 30.89% (59/191) through serology and molecular detection respectively. All the 59 positive samples were amplified for PCV2 whole genome, out of which 7 representative samples were sequenced, annotated and submitted in GenBank. The strains from this study formed a separate cluster in the phylogenetic tree resulted from the inter-genotypic recombination between PCV2c and PCV2d. This novel cluster was found to be highly dissimilar and distant from the previously reported recombinant strains from China and India, derived from the recombination of PCV2a and PCV2b (Cai et al., 2012; Anoopraj et al., 2015). Recombination along with mutation is considered to be substantial for genetic variability and evolution (Ma et al., 2007; Olvera et al., 2007). ORF2 codes for the structural and virulence associated protein – Cap (capsid) protein (Nawagitgul et al., 2000; Olvera et al., 2007), which is also the main antigenic determinant of the virus (Finsterbusch and Mankertz, 2009; Nawagitgul et al., 2000) playing key role in virus attachment interacting with heparin sulphate receptor on target cell (Misinzo et al., 2006). In this study, a possible recombination event was detected in the ORF2 sequence through Simplot and Bootscan analysis. A representative isolate (MH509736) was selected, as the query sequence, EU148505, KJ729075 as a putative parent and AY193712 PCV1 as out-group to evaluate possible recombination in the representative isolate (MH509376). Analyzed results strongly indicated a possible recombination event with potential breakpoints at 429 nt and 521 nt within the cap gene.

Crystal structure of the PCV2 capsid protein indicate four antibody recognition domains; A: 51 to 84; B: 113 to 132; C: 169 to 207; D: 228 to 233. On a more detailed note, there are immune-dominant decoy epitope binding sites embedded at position 70-asp, 71-Met, 77-Asn and 78-Asp within the A domain; 113:Glu, 115:Asp and 127:Asp within the B domain and 203:Glu, 206:Ile and 207:Tyr within the C domain; necessary for antibody recognition (Khayat et al., 2011). Specific substitutions at capsid protein on immunoreactive domain (A to D) results in changes in virus neutralization and pathogenicity (Saha et al., 2012). Multiple sequence alignment of the amino acid sequence represented

the conserved and variable residues of PCV2 capsid protein of the representative isolate (MH509736) with the consensus sequence of established crystallographic structure of 3R0R:A that was treated as a reference. Comparison with the consensus sequence, PCV^{CS} (PDB ID: 3R0R:A) reveals substitution of (I58 → V), (R60 → A), (K64 → T) in the A- domain, (S122 → T) in the B-domain and (L186 → M) (A191 → T), (I207 → K) in the C-domain. No substitution was encountered in the D-domain (Fig. 4). The secondary structure- wiring diagram was drawn to determine the stability and helical characteristics of the capsid protein. The representative isolate (MH509736) indicated motifs of the capsid protein as 3 sheets, 1 beta hairpin, 5 beta bulges, 13 strands, 2 helices, 34 beta turns and 8 gamma turn, while the motif of the PCV2 consensus sequence (PCV^{CS}) (PDB ID: 3R0R: A) indicated 3 sheets, 1 beta hairpin, 5 beta bulges, 13 strands, 3 helices, 14 beta turns and 2 gamma turn (Fig. 5). Apart from variability in motifs with the consensus sequence, the representative isolate (from this study) indicated presence of some interior spaces connected with the protein surrounding in the form of tunnels (Fig. 6D). The tunnel was calculated to possess 1.44 Å radius comprising of 2 positive, 1 each of neutral, aliphatic and aromatic residues. Thus the tunnel is seen as an interface region connecting each chain in 4D (Fig. 6C) thus potentially influencing protein reactivity.

The interaction of PCV2 with host generally initiates with viral attachment and entry into the host cells (Nauwynck et al., 2012) and it is thought to develop PMWS by alteration of cellular immune responses of pigs (Kekarainen et al., 2010). The pathways of innate and acquired immunity required for specific disease tolerant traits are influenced by genetic variation of the host (Lunney and Chen, 2010). Alteration in the immunogenic domains on capsid protein of PCV2 plays a crucial role in PCV2 pathogenesis (Khayat et al., 2011). Several *in vivo* experimental studies explained that amino acid changes in capsid protein could lead to a difference in PCV2 pathogenesis (Fenaux et al., 2004; Opriessnig et al., 2006). It has also been reported that motif-2b of capsid gene plays an important role to increase the virus replication as well as pathogenesis as compared to motif-1 (motif-1a and 1b) (Cheung et al., 2011, Allemandou et al., 2011). The possible pathogenicity of the presently circulating PCV2 strain due to amino acids substitution is scope for further study. Furthermore, the detection of a recombinant event with changes in critical viral capsid domains could necessitate enhanced surveillance measures regarding emergence of genomic variants of PCV2 with concomitant changes in pathogenicity.

5. Conclusion

The present study indicates that the prevalence of PCV2 in the north eastern part of India is fairly high. Identification of a recombinant outlier coupled with protein level changes in the capsid could point out to a high level of molecular variation in the PCV2 genotypes circulating in the region. Hence, an enhanced surveillance and monitoring for this virus is needed in terms of detecting molecular variants and also enhance virulence bearing genotypes.

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Conflict of interest

The authors do not have any conflict of interest.

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