Incidence of porcine circovirus-genotype 2d in swine raised under extensive management systems

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Abstract

Porcine circovirus-2 (PCV2) is a widespread pathogen in swine, characterized by the presence of multiple genotypes that are linked to a wide range of clinical and subclinical manifestations. The present study aimed to investigate the causative agent of mortality in pigs reared under extensive system of management in the Karur region of Tamil Nadu during April'2022. Samples like liver, lung, lymph node, heart swabs were collected from the necropsied cases in sterile container for molecular detection of Porcine circoviruses 1, 2 and 3 by PCR. The samples tested negative for both Porcine Circovirus 1 (PCV1) and PCV 3. However the presence of porcine circovirus 2 was identified by amplification of its ORF1 gene using specific primers. For further confirmation, sequencing of PCR product of ORF1 gene of PCV2 was carried out. The sequences were closely clustered with previously reported PCV2 sequences available in the NCBI database. The sequences of ORF1 gene of PCV2 showed 100 % similarity with the other PCV2 sequences of various countries. For genotyping of PCV 2, sequencing of the ORF2 gene of PCV2 was carried out using specific primers. The ORF2 gene sequences of PCV2 showed more than 99 % similarity with 2d genotype of porcine circovirus sequences of various countries. The present study revealed that the PCV2d genotype is circulating among free-ranging pigs and is responsible for cases of mortality.

Keywords: Porcine circovirus 2, PCR, Genotyping, PCV2d, Phylogenetic analysis

Introduction

Pig farming plays an important part of the livestock sector and contributes pork as one of the major commodities traded worldwide. Nowadays there was visible increase in piggery farms across the country which accounts for about 1.7% total livestock population according to the 20th livestock census. Among the pig population in India, non-descript pigs were contributed around 79.03% of the pork production whereas crossbreds and exotic breeds were contributed around 20.97% of the pork production as per Indian Livestock Census 2019. Porcine circovirus 2 (PCV2) has been classified under family Circoviridae and genus Circovirus. PCV2 causes a constant threat to the pig farming communities because of its associated disease known as Porcine circovirus-associated disease (PCVAD) (Segales, 2012, Mandal et al., 2012). The PCV2 virion is naked and icosahedral in nature which contains single stranded circular DNA comprising of 1767–1768 nucleotides long. The circular DNA is encoded with three open reading frames viz. ORF 1, ORF 2 and ORF 3. The ORF 1 is located in the clock wise position of the genome which encodes two replication associated proteins namely rep and rep'. The ORF 2 encodes major capsid protein which is immunogenic in nature and positioned in the counter clockwise of the Ori. The ORF 3 of PCV 2 is encoded by apoptotic protein which is coincided completely in ORF 1 in the reverse direction (Bao et al., 2018). The ORF 2 gene plays an important role for differentiation of genotypes of PCV 2 as well as formulation of vaccines due to its immunogenic nature (Olvera et al., 2007). At present, eight genotypes of PCV 2 were distinguished that is ranging from PCV 2a to PCV 2h by analyzing the ORF 2 genome of PCV 2 (Franzo and Segalé, 2018). PCV 2a was considered to be the most prevalent genotype in the year ranging from 1996 to 2000. Then, the genotype was shifted from PCV2a to PCV2b noticed in the later months of the year 2000 (Xiao et al., 2015). The genotype shift from PCV 2b to PCV 2d was reported in the year 2012 and increases rapidly (Xiao et al., 2015; Harmon et al., 2015; Bao et al., 2018). The several studies on PCV2 genotype documented that the different genotypes of PCV 2 is emerging among the pig populations (Bao et al., 2018; Dupont et al., 2008). The objective of this study is to identify the genotype of porcine circovirus circulating among free ranging pigs in order to formulate targeted control strategies which are essential for sustainable pig farming. In this study, PCV 2d genotype was detected in the pigs reared under extensive system of management in Tamil Nadu by molecular characterization of ORF 2 gene of PCV 2.

Materials and methods

Sample collection

The non-descriptive pigs were reared in LN Chathiram, Karur (10.96° N Latitude and 78.08° E Longitude) under extensive system of management. A sudden death was reported in very young pigs during the month of April 2022. Clinical examination was carried out in the two terminally ill pigs and the clinical signs observed were anorexia, lethargy, fever, congested conjunctival mucous membrane, respiratory distress, expiratory dyspnoea, nasal discharge, ocular discharge, perineal soiling indicating diarrhea, severe dehydration and lateral recumbency. A post mortem investigation was carried out into the acute mortality of three very young pigs. The post mortem examination of necropsied pig carcass revealed consolidation of lungs indicating pneumonic changes, normal liver, kidney, enlarged and haemorrhagic submandibular lymph nodes and few congested areas in the stomach mucosa. Samples such as whole blood and organs were collected from the ailing and dead animals respectively.

Bacteriological examination

The heart blood swabs and lung swabs were inoculated into different culture plates such as Brain heart Infusion agar, 5% sheep Blood agar and MacConkey agar. The plates were incubated aerobically for 24 hours at 37^0C. The colonies were subjected to gram staining and routine biochemical tests such as IMVIC tests, sugar fermentation tests, catalase and oxidase test were carried out.

DNA extraction

The DNA was extracted from pooled tissue samples using a QIAamp DNA mini kit (Qiagen, Germany) as per the manufacturer's instructions, quantified with a Nanodrop spectrophotometer and stored at -20^0C for further operations. The PCR was carried out for PCV1, PCV2 and PCV3 by using specific primers as shown in Table 1. The PCR reaction mixture is composed of 10 µl master mix (2x), 1 µl each forward and reverse primer (10 pmol/ µl), 5 µL deionized water and 3 µl extracted DNA. The thermal conditions for PCV1, PCV2, PCV3 (Rep/Cap genes): Initial denaturation at 95^0C for 4 min; 35 cycles of 94^0C for 30 seconds (denaturation), 55^0C for 40 seconds (annealing), 72^0C for 60 seconds (extension) and final extension at 72^0C for 10 min. The thermal conditions for PCV2 (ORF2 gene): initial denaturation at 94^0C for 2 min, 40 cycles at 94^0C for 30 seconds, 60^0C for 30 seconds, 72^0C for 1 min, and final extension at 72^0C for 7 min. The analysis of the PCR products was carried out in 1.5 percent agarose gel stained with ethidium bromide (0.5µg/ml) and documented.

Sequencing and phylogenetic analysis

The positive PCR products of ORF1 and ORF2 gene fragments of PCV2 in the present study were sequenced by the Sanger dideoxy sequencing method in an automated sequencer. BioEdit software was used for

sequence assembly and editing. The ORF1 and ORF2 sequences of this study were deposited at GenBank and accession numbers viz. PV177148 and PV177149 were obtained respectively. Homology was searched using the NCBI BLAST with the reference sequences available in this database. The multiple sequence alignment (Clustal W) was done by Mega X1 software to generate sequence analysis data.

The phylogenetic tree for ORF1 and ORF2 gene fragments of PCV2 was created by Neighbour Joining (NJ) approach using bootstrap values (1000 replicates) and distance in Mega X1 software in order to assess the relationship between the sequences.

Results

Post mortem examination

The gross post examination of necropsied pig carcass revealed consolidation of lungs indicating pneumonic changes, normal liver, kidney, enlarged and haemorrhagic submandibular lymph nodes and few congested areas in the stomach mucosa**.**

Bacteriological examination

E. coli could be identified by culture from heart blood and lung swabs from necropsied pig by conventional methods. Hematological analysis revealed lymphopenia and marked thrombocytopenia. Serum biochemical analysis revealed mild hypoglycemia, mild hypoproteinemia, and elevated aspartate transferase (AST), creatinine kinase (CK), and Calcium values.

Polymerase chain reaction

The pooled tissue samples such as liver, lung and lymph nodes from the necropsied cases were found to be negative for PCV1 and PCV3 by PCR. However, the pooled tissue samples were found to be positive for PCV2 by amplifying the ORF1 gene using specific primers and yielded an amplicon size of 505 bp product specific to PCV2. Further, PCR was carried out for genotyping of PCV2 by using ORF2 gene specific primers and yielded an amplicon size of700 bp in length. The PCR products of both ORF1 and ORF2 genes of PCV2 were subjected to DNA sequencing. The representative sequences of ORF1 and ORF2 genes were submitted to GenBank and obtained accession numbers MT350794 and MT350795 respectively.

Table 1: Details of primers used in this study for detection of Porcine circoviruses

Target gene	Primer sequence (5' to 3')	Amplicon size	References
PCV1 Rep	PCV1-FP GAAAGTGAGCGGGAAGAT PCV2-RP CTGATTGCTGGTAATCAA	310 bp	Yang *et al*., 2019
PCV2 ORF1	PCV2-FP CACATCGAGAAAGCGAAAGGAAC PCV2-RP TGCGGGCCAAAAAAGGTACAGTT	505 bp	
PCV3 Rep, Cap	PCV3-FP AGCAGTGCTCCCCATTGA PCV3-RP TGGGCCCGACCAAATCCGG	1021 bp	
PCV2 ORF2	PCV2-FP CCATGCCCTGAATTTCCATA PCV2-RP ACAGCGCACTTCTTTCGTTT	800 bp	Takahagi *et al*., 2008

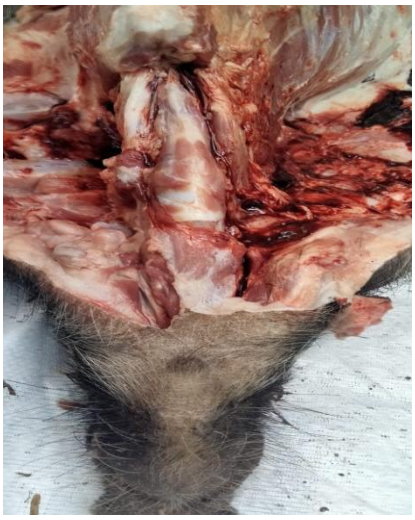

Fig 1. Postmortem examination of dead pig

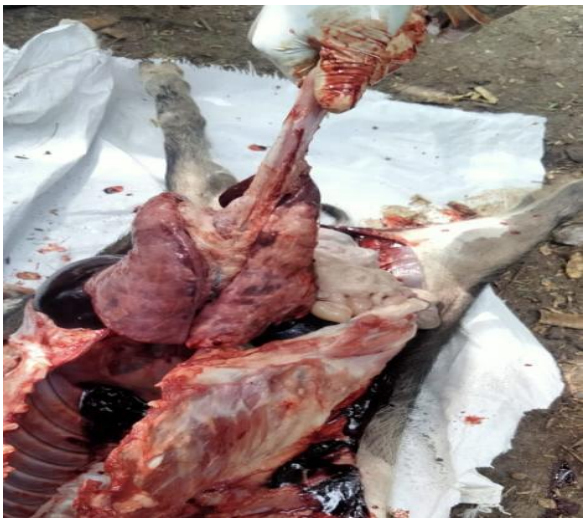

Fig 2. Consolidation of lungs indicating pneumonia

Table 2: The haematological values of two terminally ill pigs

Sl. No.	Parameters	Affected Pig
1	RBC ($x10^6$/µl)	5.76
2	Hb (g/dl)	9.4
3	PCV (%)	34.2
4	WBC ($x10^3$/ µl)	20.6
5	Neutrophils (%)	76
6	Lymphocytes (%)	18
7	Eosinophils (%)	-
8	Monocytes (%)	6
9	Platelets ($x10^3$/ µl)	65000

Table 3: Serum biochemical parameters of two terminally ill pigs

Sl. No.	Parameters	Affected Pig
1	Glucose (mg%)	63.0
2	Total Protein (g%)	7.16
3	Albumin (g%)	2.84
4	Bilirubin (mg/dl)	0.86
5	BUN (mg/dl)	35.0
6	Creatinine (mg/dl)	0.99
7	ALT (U/L)	28.7
8	AST (U/L)	101.6
9	ALP (U/L)	117.0
10	CK (U/L)	780.0
11	Calcium (mg/dl)	15.9
12	Phosphorus (m/dl)	10.0
13	Sodium (mEq/L)	129.0
14	Potassium (mEq/L)	6.5
15	Chloride (mEq/L)	87.0

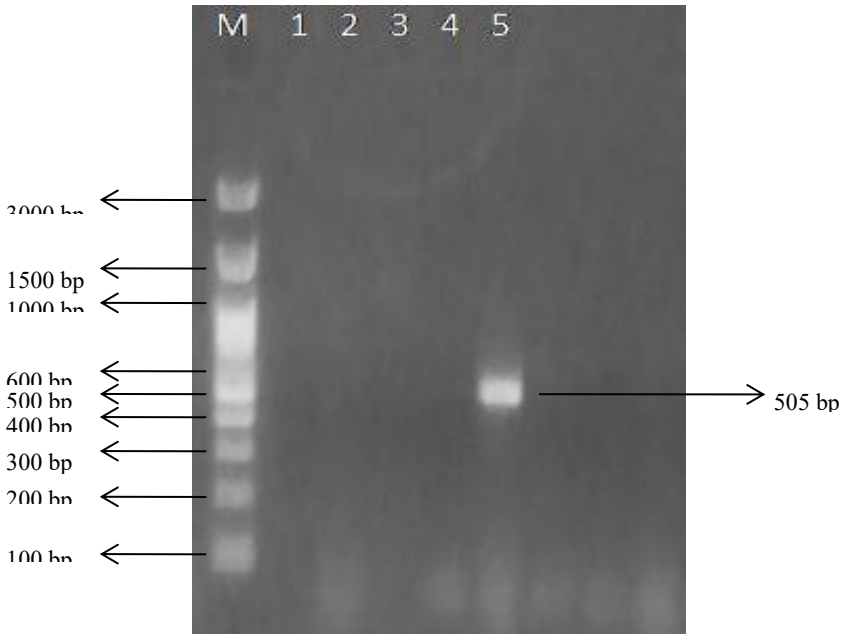

Fig 3: Agarose gel electrophoresis showing amplicons specific to PCV-2 and negative for PCV1 and PCV3 (Lane M: DNA ladder 100bp-3000bp; Lane 1&2: No template control; Lane 3: PCV1; Lane 4: PCV 3; Lane 5: PCV2)

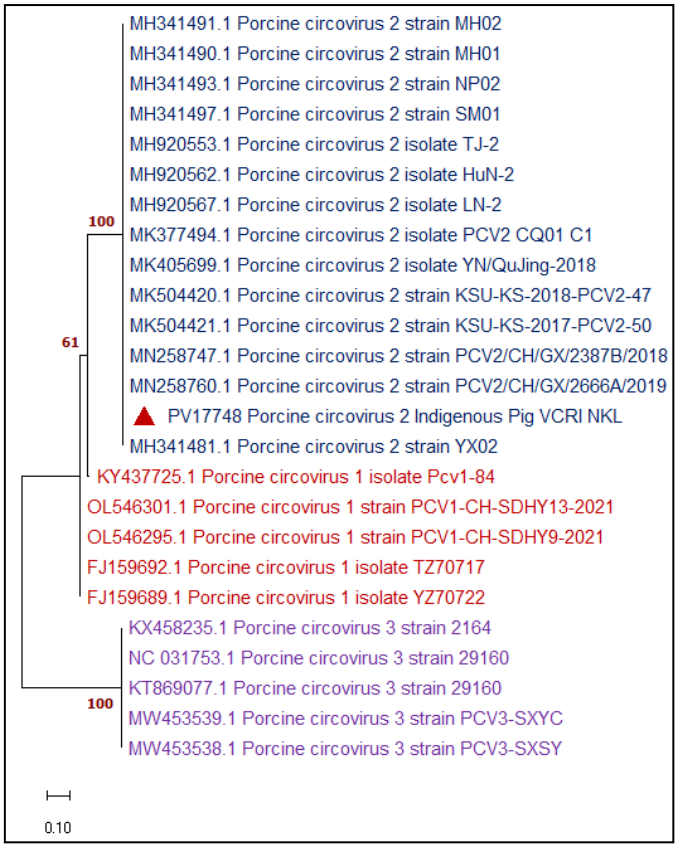

Fig 4: Phylogenetic analysis of ORF1 gene sequence of PCV2 which was drawn by Neighbour Joining algorithm with 1000 bootstrap replicates in Mega XI software.

DNA sequencing

The representative ORF1 gene sequence was subjected to multiple sequence alignment using the reference sequences published in the NCBI website. The ORF1 gene sequence was found to be similar to PCV2 virus by analyzing the sequences at the nucleotide as well as amino acid level. Likewise ORF2 gene sequence generated in the present study were aligned using the reference sequences of various PCV2 genotypes. The ORF2 gene sequence of this study was found to be more than 99 % similarity to that of PCV2d genotype of other country sequences published in the NCBI website.

The ORF2 gene sequence generated in this study had mostly identical nucleotide sequence with PCV2d genotype however had nucleotide mutations at residues 1034 (C→T) with Accession No.KX668492, 1378 (C→G) with Accession Nos. MK347371 and MK347364, 1414 (G→T) with Accession Nos. KX668491, KX668492, MK347371 and MK347364 in comparison to other PCV2d genotypes.

Phylogenetic analysis

Phylogenetic analysis of ORF1 gene sequence of this study was clustered with other PCV2 sequences available in the NCBI database. PCV1 and PCV3 sequences retrieved from NCBI database were clustered in separate distinct group (Figure 4).

Whereas, phylogenetic analysis of ORF2 gene of PCV2 sequences retrieved from NCBI clearly distinguished the different genotypes of PCV2 and ORF2 gene sequence generated in the present study was clustered with PCV2d genotypes of other published sequences (Figure 5).

Discussion

Porcine circovirus 2 is considered to be one of the important pathogens which causes great economic losses due to mortality as well as lowered production performances and affect the livelihood of the pig farming community throughout the world. PCV 2 was reported to be affect all age groups but the prevalence rate was more than 85% in the age group ranging from 85 to 112 days old (Chen *et al*., 2023). In the present study, acute death was observed in very young non-descriptive pigs reared under extensive system of management.

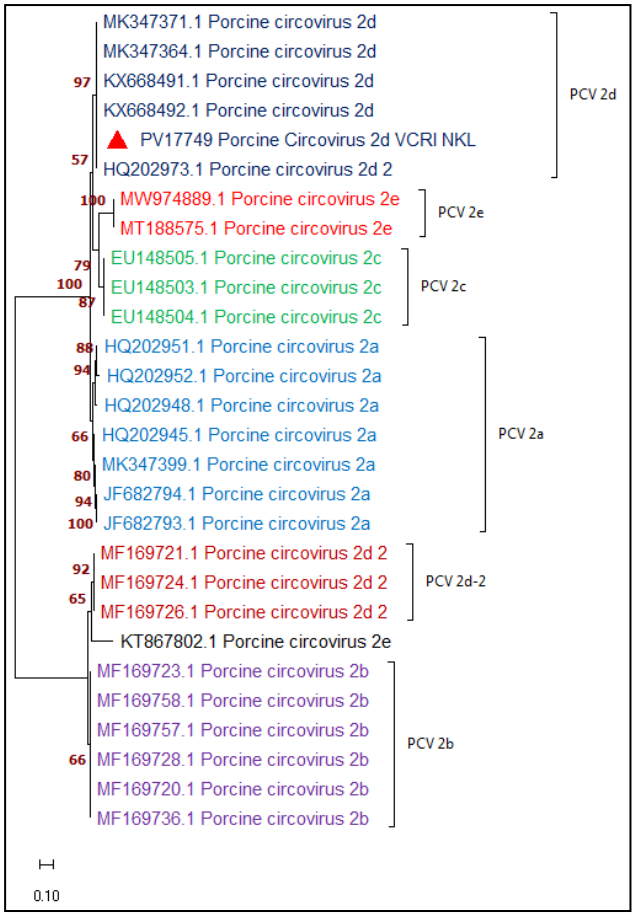

Fig 5: Phylogenetic analysis of ORF2 gene sequence of PCV2 constructed by Neighbour Joining algorithm with 1000 bootstrap replicates in Mega XI software.

The clinical manifestation of diseased pigs was fever, congested conjunctival mucous membrane, dyspnoea, diarrhea and lateral recumbency. The gross lesions such as pneumonia, enlargement as well as haemorrhagic submandibular lymph nodes and congested mucosa in the stomach were observed during necropsy. In the present study, altered hematological and biochemical parameters viz. marked lymphopenia, thrombocytopenia, hypoglycemia, hypoproteinemia and elevated levels of AST, CK and calcium were observed. The findings were consistent with the previous report (Rajesh *et al*., 2020). In this study, *E. coli* could be identified by culture from heart blood and lung swabs from necropsied pigs by conventional methods. The PCV2 infection is mostly co-infected with other viral and bacterial pathogens viz. PRRSV, PPV, *M.hyopneumoniae* and other pathogens (Opriessnig and Halbur, 2012; Segalés et al., 2013)

In this study, PCV2 was detected in the tissue samples by amplification of the portion of ORF1 gene using gene-specific primers. The ORF1 gene was reported to be highly conserved and is required for viral replication by encoding replicase protein. The molecular results of this study were in agreement with previous findings for detection of PCV2 in tissue samples using ORF1gene.

For further confirmation of PCV2 infection in pigs, it was confirmed by nucleotide sequencing of partial ORF1 gene of PCV2. The nucleotide sequence generated in this study was found to be 100 percent identity with other published sequences of PCV2. Further phylogenetic analysis of ORF1 gene revealed that the sequence obtained in this study was clustered with other PCV2 sequences. The tree was constructed by maximum likelihood method with 1000 bootsrap value along with distance. The sequence generated in this study was closely clustered with the group comprising PCV2 strains of other countries viz. China and the USA.

Further, PCR was carried out by using primers specific to ORF2 gene of PCV2 to determine the genotype of PCV-2 strains associated with this infection. Though the ORF2 gene is immunogenic and encodes major capsid protein of PCV2, it is being commonly used for differentiation of genotypes of PCV2 by many reports. In the present study, nucleotide sequencing of a representative sample was done to detect the genotype of PCV2 infection in the native pigs responsible for mortality. The nucleotide sequence of ORF2 gene with 800bp length of PCV2 from indigenous pigs was compared with 27 PCV2 sequences of different genotypes retrieved from NCBI. It has been observed that a nucleotide sequence of PCV2 was found to be similar to that of PCV2d genotype after

analyzing the sequences with other published sequences. There were three changes at nucleotide positions 1034 (C→T), 1378 (C→G) and 1414 (G→T) were observed with other PCV2d sequences.

The phylogenetic tree was constructed by maximum likelihood method with 1000 bootsrap value along with distance. The PCV2 sequence generated in this study was positioned in the group comprising PCV2d genotypes of Chinese strains / isolates which clearly indicating that PCV2 belongs to PCV2d genotype. Further, grouping is distinct with groups comprising of strains according to their genotypes. This indicates that variation in ORF2 gene has relevance to the molecular genotyping of the PCV2 infection in pigs.

Conclusion

It is concluded that PCV2 infection is responsible for clinical disease as well as mortality in the non-descript pigs determined by PCR using ORF1 gene of PCV2. Further sequencing of ORF2 gene of PCV2 revealed that the PCV2d genotype is responsible for the infection in the indigenous pigs.

Conflict of interest

The authors declare that no conflict of interest.

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