MOLECULAR CHARACTERISATION OF *PORCINE CIRCOVIRUS 2* AND DEVELOPMENT OF A REAL-TIME POLYMERASE CHAIN REACTION FOR DETECTION OF THE VIRUS

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I hereby declare that this thesis, entitled **"Molecular characterisation of *Porcine circovirus 2* and development of a real-time polymerase chain reaction for detection of the virus"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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ABBREVIATIONS

BLAST	-	Basic local alignment search tool
bp	-	base pair
℃	-	Degree Celsius
Cap	-	Capsid
CSF	-	Classical swine fever
DNA	-	Deoxyribonucleic acid
FAO	-	Food and Agricultural Organization
g	-	g force
h	-	hours
mg	-	milligram
mL	-	millilitre
µL	-	microlitre
µg	-	microgram
µM	-	micromolar
MEGA	-	Molecular evolutionary genetics analysis
min	-	minutes
ng	-	nanogram
NFW	-	Nuclease free water
NTC	-	No template control
ORF	-	Open reading frame
PCR	-	Polymerase chain reaction
PCV	-	Porcine circovirus
PCV2-SD	-	Porcine circovirus 2 - systemic disease
PCVAD	-	Porcine circovirus associated disease
PDNS	-	Porcine dermatitis and nephropathy syndrome
PMWS	-	Post weaning multisystemic wasting syndrome
PPV	-	Porcine parvovirus
PRDC	-	Porcine respiratory disease complex
PRRSV	-	Porcine respiratory and reproductive syndrome virus
pmol	-	picomole
qPCR	-	quantitative PCR
RT-PCR	-	Real-time polymerase chain reaction
Rep	-	Replicase
sec	-	seconds
TBE	-	Tris-borate-EDTA
UV	-	Ultraviolet

Introduction

1. INTRODUCTION

Agriculture accounts for up to 15 per cent of the gross domestic product (GDP) in higher-income countries, whereas in low-income countries the figure is around 30 per cent (Van der Waal and Deen, 2018). Food security to communities is a major challenge in the expanding world due to increased urbanization, ever-growing population, and climate change which often impart its consequence over animal source food and thereby food production globally. In a developing world, an enhanced need for animal source food provides numerous livelihood and employment opportunities in the livestock sector. Livestock products also play a major role in providing critical micronutrients, which are required to improve the nutritional status of household members and generate income (Randolph *et al*., 2007).

The livestock sector plays a crucial role in the Indian rural economy. According to Department of Animal Husbandry and Dairying, the contribution of the livestock sector to the total GDP of India was around 4.11 per cent during 2018-2019 and the total value of output from the livestock sector in the agriculture, forestry, and fishery sector was about 25.6 per cent. It promises food security by ensuring safe and wholesome food to even vulnerable sections of the nation and acts as a pillar in crises such as agricultural failure (Mukesh *et al*., 2022). According to the Economic Survey report 2021-2022, in India, livestock farming serves as a chief source of doubling farmers' income.

Pig rearing is one of the major livestock farming activities in the world. Due to its high fecundity, greater feed conversion efficiency, early maturity, and short generation interval compared to other livestock species, it has a significant potential to help farmers realize a faster economic return (Delsart *et al*., 2020).

The swine population in the world is nearly 100 million, and the main countries investing in pig farming are China, the United States, and Brazil. As per the estimates of FAO in 2018, the worldwide per capita consumption of pork is 12.3

kg per year, thus making pork the most consumed meat after chicken (Rivera *et al*., 2021).

As per the 20th livestock census, the total number of pigs in India is 9.06 million, which shows a decline of 12.03 per cent over the previous census carried out in 2012, wherein the pig population was 10.29 million (20th Livestock Census, 2019). The current pig population is around 1.7 per cent of the total livestock population.

In India, the highest pig population is in Assam, followed by Uttar Pradesh and Jharkhand. Pig rearing and pork consumption are mainly limited to the Northern and North-Eastern states of India, Kerala, Punjab, Bihar, and tribal belts of Uttar Pradesh. Moreover, pork consumption is mainly concentrated in unorganized sectors such as locally reared fresh meat. To the total meat production, the share of pork is eight per cent. The unexploited nature of swine production is often contributed by numerous constraints such as underdeveloped breeding practices, unorganized production system, insufficient and improper feeding, and a lack of advances in healthcare management to control the spread of dreadful diseases and to keep infections under control (Chauhan *et al.*, 2016).

Infectious diseases compromise pig health and negatively affect the growth, productivity, and stability of the pig industry worldwide. As the swine industry has intensified and globalized, pathogens of swine have emerged and spread globally, partly due to the transportation of pigs, pork products, and by-products and feed at all levels. Infectious diseases affect livestock production directly through mortality, productivity loss, trade restrictions, diminished market value, and food insecurity. The emerging and endemic diseases of swine, which are often considered constant threats, have devastating consequences for the industry all over the world (Van der Waal and Deen, 2018).

Many infectious agents cause diseases in pigs. Common viral infections of pigs are classical swine fever (CSF), porcine reproductive and respiratory syndrome (PRRS), *porcine circovirus 2* (PCV2) infection, and porcine parvovirus (PPV)

infection. Respiratory diseases in pigs are together referred to as porcine respiratory disease complex (PRDC). The viral and bacterial infectious causes of PRDC include swine influenza virus, PRRS virus (PRRSV), PCV2, *Pasteurella multocida*, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Streptococcus suis* and *Haemophilus parasuis* (Zhu *et al*., 2021).

Porcine circovirus is an emerging swine pathogen causing immense economic losses in the global swine industry. The virus was found to be associated with a disease syndrome called post-weaning multisystemic wasting syndrome (PMWS) (Clark, 1996; Harding, 1997). *Porcine circovirus 2* is the member of genus *Circovirus* within the family *Circoviridae*. The genus Circovirus includes many 49 species of viruses including PCV1, PCV2, PCV3 and PCV4 (ICTV, 2021). Morphologically, porcine circovirus appears as small, non-enveloped, icosahedral particles of 15–20 nm in size approximately (Meng, 2012).

According to the current genotyping methodology, the molecular analysis of PCV2 isolates reveals eight different genotypes, namely PCV2a, PCV2b, PCV2c, PCV2d, PCV2e, PCV2f, PCV2g, and PCV2h (Franzo and Segalés, 2018). Infection with PCV2 is clinically characterized by wasting, paleness of the skin, respiratory distress, and occasionally, diarrhoea and jaundice in the late nursery and fattening pigs at usually 4 to 16 weeks of age (Segalés *et al.,* 2005). Affected animals displayed characteristic lesions in multiple tissues (multisystemic), mainly in lymphoid organs (Clark, 1997; Rosell *et al*., 1999). Several clinical syndromes have been linked to PCV2 infection. The most described one is PMWS, which has been referred with different names in the literature including porcine circovirosis (Rosell *et al.*, 2000b), PCV2-associated systemic infection (Opriessnig *et al*., 2007), and porcine circovirus-associated disease (PCVAD) in some cases (Carman *et al*., 2008). Other pathological conditions linked to PCV2 have been porcine dermatitis and nephropathy syndrome (PDNS) (Allan *et al*., 2000), reproductive failure (Madson *et al*., 2009a), proliferative and necrotizing pneumonia (Grau-Roma and Segalés, 2007), respiratory disease (Cheng *et al*., 2011) and enteritis (Kim *et al*.,

2004). Besides these, the virus, in association with other porcine viruses such as PRRSV, increases the severity of clinical symptoms in pigs.

Immunohistochemistry, *in situ* hybridization, indirect immunofluorescence, polymerase chain reaction (PCR), PCR along with restriction fragment length polymorphism, real-time PCR, and virus isolation are the usual methods employed for diagnosis (Allan and Ellis, 2000; Zhao *et al*., 2010).

Most studies on the genetic characterization of PCV2 are based on the Cap gene, which is the ideal marker for phylogenetic analysis because this region is considered to be the most variable region in the PCV2 genome and the same phylogenetic tree constructed based on the full genome can be reconstructed with open reading frame 2 (ORF2). However, analysis based on the complete genome is necessary, as it may help identify genetic variability, particularly in terms of recombination events (Cao *et al*., 2018).

Even though PCV2 has been reported in Kerala in 2016, no systematic study has been performed to characterize the virus and elucidate the lineage of the virus prevalent in pigs in Kerala. Also, there is an urgent need for developing sensitive diagnostic tests to detect PCV2 prevalent in Kerala. Therefore, the present study was carried out with the below-mentioned objectives:

1. Molecular characterisation of *Porcine circovirus 2* (PCV-2) based on the whole genome sequence of the virus.
2. Development of a TaqMan probe-based real-time polymerase chain reaction (RT-PCR) for detection of the virus.

2. REVIEW OF LITERATURE

2.1 HISTORY

Porcine circovirus (PCV) was first detected as a contaminant in PK-15 cell line and experimental infections of pigs with this virus did not produce clinical disease (Tischer *et al*., 1974, 1986; Allan *et al*., 1995).

Post-weaning multisystemic wasting syndrome in pigs was first identified in western Canada in 1991 (Clark, 1997; Harding, 1997). The syndrome was characterized by progressive weight loss, respiratory signs and jaundice. Similar syndromes were reported in pigs in the USA, France, N. Ireland and Spain (Daft *et al*., 1996; LeCann *et al*., 1997; Segale´s *et al*., 1997; Kennedy *et al*., 1998).

The circovirus isolates associated with wasting syndromes in pigs were reported to be significantly different from the PCV contaminant of PK/15 cell cultures (Meehan *et al*., 1997, 1998; Mankertz *et al*., 1997; Hamel *et al*., 1998) and it was suggested that these novel porcine circoviruses may represent pathogenic strains of PCV (Nayar *et al*., 1997; Allan *et al*., 1998; Ellis *et al*., 1998; Hamel *et al*., 1998; Meehan *et al*., 1998). Later, on the basis of genomic and antigenic differences, the new circovirus isolates were referred to as PCV2 and the original PCV contaminant of PK/15 cell cultures as PCV1 (Allan *et al*., 1998; Meehan *et al*., 1998).

Jacobson *et al*. (2009) reported that the earliest retrospective evidence of PCV2 infection in pigs dated from 1962 in Germany.

2.2 CLASSIFICATION

Porcine circovirus was initially classified under the newly created family *Circoviridae* along with chicken anaemia virus (CAV) and beak and feather disease virus (BFDV) of psittacine birds (Lukert *et al.*, 1995).

In order to distinguish the circovirus isolated from wasting syndromes in pigs from the contaminant circovirus, the latter was named as PCV1 and the former as PCV2 (Allan *et al*., 1998; Meehan *et al*., 1998).

Porcine circovirus 2 was classified into four genotypes: PCV2a (earlier group II) with five subtypes or clusters (2A, 2B, 2C, 2D, and 2E) PCV2b (earlier group I) with three clusters (1A, 1B, and 1C), PCV2c and PCV2d (Segales *et al.*, 2008; Guo *et al.*, 2010).

Currently, PCV2 belongs to the realm *Monodnaviria*, kingdom *Shotokuvirae*, phylum *Cressdnaviricota*, class *Arfiviricetes*, order *Cirlivirales*, family *Circoviridae* and genus *Circovirus* and eight different genotypes, namely PCV2a, PCV2b, PCV2c, PCV2d, PCV2e, PCV2f, PCV2g, and PCV2h have been reported (Franzo and Segalés, 2018, International Committee on Taxonomy of Viruses, 2021).

2.3 VIRION PROPERTIES

Tischer *et al*. (1982) reported that the PCV virion had an icosahedral capsid of 17 ± 1.3 nm diameter with a single circular stranded DNA.

Breitbart *et al*. (2017) reported that the PCV virion was non-enveloped, round, and that it had T=1 icosahedral symmetry. Virions are made up of 60 capsid subunits.

2.3.1 Viral genome and proteins

Allan *et al*. (1995) reported that the genome of PCV2 was approximately 1.7 kb in size and has ORFs.

Mankertz *et al*. (1998) reported that the 945 nucleotide ORF1 encoded for the ‘Rep’ protein responsible for virus replication.

Nawagitgul *et al*. (2000) reported that the 702 nucleotide or 705 nucleotide ORF2 encoded for the structural capsid protein ‘Cap’, which was involved in eliciting the host immune response. The N-terminus of the ‘Cap’ protein contained residues rich in basic amino acids, which might be involved in forming the interior surface of the virion and interacting with the negative charges of genomic DNA during virus assembly (Meehan *et al.*, 1998; Lekcharoensuk *et al.*, 2004).

Liu *et al*. (2005) reported that the circular genome also contained a small ORF3 that codes for a protein, widely implicated in apoptosis.

The molecular mass of Cap, Rep, and ORF3 proteins were reported to be 27.8 kDa, 35.8 kDa, and 11.9 kDa respectively (Nawagitgul *et al.,* 2000; Lv *et al.,* 2014; Liu *et al.,* 2006)

Timmusk *et al*. (2006) reported that the Rep protein could interact with cellular filament protein and transcriptional regulator c-myc and could also interact with Cap protein, indicating that Rep proteins are important in PCV2 infection and pathogenicity.

Meng *et al*. (2010) reported that especially the N-terminus of Rep (RepN), was conserved and immunogenic.

Wu *et al*. (2012) reported that the PCV2 Cap contained sole cysteine residue at amino acid (AA) 108 (Cys 108).

Hua *et al*. (2013) reported that Rep proteins likely contributed to viral pathogenicity *in vivo*.

Lv *et al.* (2014) reported that ORF1 gene of PCV2 coded for eight replication-associated proteins, including two major products (designated Rep and Rep0) and six minor products (designated Rep3a, Rep3b, Rep3c, NS515, NS672, and NS0)

Kweon *et al*. (2015) reported that Cap protein was relatively more variable than the other proteins of PCV2.

2.3.2 Physio-chemical properties

Allan *et al*. (1994) reported that the porcine circoviruses were resistant to inactivation when exposed to chloroform, solutions with pH 3, and a temperature of 70°C.

Royer *et al.* (2001) reported that PCV2 is resistant to lipid dissolving disinfectants like alcohol, chlorhexidine, iodine, and phenol. But alkaline

disinfectants, quaternary ammonium compounds, and oxidizing agents inactivated the virus (Martin *et al.,* 2008)

Welch *et al.* (2006) studied the effects of pasteurization on the viability of PCV2 and opined that it was quite resistant to temperatures of 60℃ for 24 h, and that the titre dropped markedly when treated at temperatures above 70℃.

The virus was more labile when heated in liquid cultures than when dried, and freeze-dried PCV2 was not inactivated at temperatures of 120℃ (Welch *et al.*, 2006)

The study conducted by O'Dea *et al*. (2008) showed that the PCV2 was completely inactivated after treatment at 80℃ and above for 15 min.

2.3.3 Recombination in PCV2

Nawagitgul *et al.* (2000) and Misinzo *et al.* (2006) reported that recombination event with breakpoints in the ORF2 region was of notable importance as Cap protein determines the antigenicity and virulence.

According to Olvera *et al.* (2007) recombination was an important mechanism of PCV2 evolution that accounted for genetic variability and mutation. They also reported that the Rep gene was the hot spot for inter-genotypic recombination.

Inter-genotypic recombinant strains with potential breakpoints located within the ORF2 region of the PCV2 genome have also been reported recently in the USA and China (Cheung, 2009; Cai *et al.*, 2012; Huang *et al.*, 2013).

Cadar *et al*. (2012) recorded evidence for inter- and intra-genotypic recombination of PCV2 strains.

Anoopraj *et al.* (2015) reported the emergence of recombinant strains arising out of natural inter-genotypic recombination between PCV2a–2C and PCV2b–1C genotypes in India.

2.4 DISTRIBUTION

2.4.1 Global distribution of PCV2 in Pigs

In the early 1990s, the first known epidemic and description of the porcine multisystemic wasting syndrome occurred in fairly robust farrow-to-finish swine farms in Northeastern Saskatchewan, Western Canada, North America (Clark, 1996; Harding, 1997; Ellis *et al.,* 1998)

In the following years, PMWS and other PCVADs were observed in many countries including European countries such as Spain (Segalés *et al.,* 1997), France (LeCann *et al.,* 1997), Hungary (Dán *et al.,* 2003), and United Kingdom (Allan *et al.,* 1998; Grierson *et al.,* 2004); South America countries such as Brazil (De Castro *et al.,* 2007) and Uruguay (Ramos *et al.,* 2012); Asian countries such as Korea (Choi *et al.,* 2000) and China (Yang *et al.,* 2008); Oceania, Australia (Raye, 2004); the Caribbean, Cuba (Pérez *et al.,* 2010); the Middle East, Israel (Pozzi *et al.,* 2008); and African countries such as South Africa (Drew *et al.,* 2004) and, recently, Uganda (Ojok *et al.,* 2013).

Porcine circovirus 2 infection was also reported from several Asian countries like China, Korea, Japan, Malaysia, Thailand and Indonesia. (Tantilertcharoen *et al.,* 1999; Jaganathan *et al.,* 2011; Rose *et al.,* 2012)

Dupont *et al*. (2008) reported that both PCV2a and PCV2b were present throughout the world, with PCV2b being the predominant genotype found since 2003, and that the third type, PCV2c was reported only in Denmark from the archived material. Later, Guo *et al*. (2010) reported the presence of PCV2d isolates in China.

2.4.2 Prevalence of PCV2 in Pigs in India

In India, PCV2 was first detected in 2006 in cases of PMWS-associated PCV2 systemic disease (PCV2-SD) (Kumar *et al.,* 2006). The virus was also detected along with porcine parvovirus in crossbred pigs (Sharma and Saikumar, 2010).

Anoopraj *et al.* (2015) reported that both PCV2a and PCV2b have been identified in the Indian pig population.

Bhattacharjee *et al*. (2015), reported the complete genome sequence of PCV2a and PCV2b from north eastern states of India.

A prevalence rate of 11.56 per cent for PCV2 was reported by Pegu *et al*. (2017), in 1064 serum samples of pigs collected from north eastern states of India.

In a similar study by Mukherjee *et al.* (2018) in Meghalaya, a mean positivity of 18.94 per cent was reported for PCV2.

In another study, Mukherjee *et al*. (2018) reported the seroprevalence of PCV2 in Meghalaya as 80.8, 79.1 and 96.2 per cent in 2014, 2015, and 2016 respectively.

A comprehensive study of PCV2 and its incidences in north east India from 2011 to 2017 detected a mean seropositivity of 31.27 per cent (Barman *et al.*, 2018).

Rajesh *et al*. (2019), reported a 49.35 per cent prevalence of PCV2 in samples collected from all the north eastern states of India.

2.4.3 Prevalence of PCV2 in Pigs in Kerala

The presence of PCV2 infection in Kerala was first reported by Aishwarya (2016) from North Kerala. In the subsequent year, the presence of PCV2 infection among piglets in Thrissur district of Central Kerala was reported (Keerthana *et al.*, 2017).

2.5 EPIDEMIOLOGY

Allan and Ellis (2000) reported that infection with PCV2 was frequent in domestic pigs. They also opined that PDNS was more prevalent than PMWS in England, Ireland, and elsewhere.

It has been reported that PMWS has been diagnosed in pigs in five continents (Grau-Roma *et al.*, 2011).

Chae (2005) reported that sporadic disease occurs in North America while in Asia and Europe, PMWS occurs both in endemic and epidemic forms.

2.5.1 Host

Rose *et al*. (2003) conducted a case-control study in France between 2000 and 2001 and discovered that pig farms with PCVD had a greater proportion of pigs with PCV2 antibodies at 13 weeks of age.

Infection in finishers appeared to occur at 10–15 weeks of age when they became viraemic (Larochelle *et al.,* 2003; Shibata *et al.,* 2003) and finishing-age pigs can also get infected with PCV2 (Puvanendiran *et al.*, 2011),

According to Sibila *et al.* (2004), young animals (1–10 weeks old) and those between 16 and 20 weeks in PCVD-affected farms had increased viral prevalence in the nasal cavity and serum.

McKeown *et al*. (2005) reported that the PCV2-SD (PCV2-systemic disease) or PCVAD affected grower pigs from 7–16 weeks of age presumably when maternal antibody protection fades away.

Rose *et al*. (2005) conducted a cohort field study and reported that the offspring of pure-bred Pietrain boars were not better protected against PCVD than pigs produced with the usual cross-bred boars. Moreover, in this study, the PCV2 course of infection was similar in the offspring of different genetic origins. In contrast, lower mortality due to PCVD in pure or cross-breed Pietrain in comparison with Large White–Duroc cross pigs has also been reported (Lopez-Soria *et al.,* 2011).

Experimental studies conducted by Opriessnig *et al.* (2006) concluded that Landrace pigs were found to be more susceptible to PCV2-induced problems than Large White and Duroc. Later, Opriessnig *et al.* (2009b) reported that the Landrace pigs were more susceptible than Pietrain pigs when comparing the severity of PCV2-associated microscopic lesions.

Porcine circovirus 2 can also infect breeding sows, newborn piglets, and piglets less than 10 weeks of age (Grau-Roma *et al.*, 2009; Shen *et al.*, 2010; Patterson *et al.*, 2011).

2.5.2 Transmission

2.5.2.1 *Direct transmission*

Welch *et al.* (2006) reported that PCV2 was stable in the environment. Direct contact between animals was considered to be the most efficient form of transmission (Andraud *et al*., 2008).

It has been reported that PCV2 is shed through colostrum, milk, faeces, and nasal and oral secretions of infected pigs (Fort *et al.*, 2008; Rose *et al.*, 2012)

In an experimental study by Kristensen *et al*. (2007), airborne transmission from diseased to healthy pigs was proven to be an alternate route of transmission of PMWS infection.

Some case studies reported abortions associated with myocarditis in foetuses due to PCV2 (Brunborg *et al.*, 2007; West *et al.*, 1999).

Schmoll *et al.* (2008) reported that PCV2 DNA was shed in semen. The age of the boars shedding PCV2 DNA in semen ranged between 35.9 and 71.0 weeks, and shedding occurred during a period of up to 27.3 weeks.

2.5.2.2 *Indirect transmission*

Lörincz *et al.* (2010) reported that the PCV2 could be found at quite high prevalence levels in dead mice and rats in field conditions, which raises the possibility of indirect transmission and persistence on a farm site.

Rose *et al*. (2012) reported that PCV2 can potentially be transmitted through several indirect ways, including oral, aerosol, fomites, and vaccination routes.

2.5.3 Persistence

Pigs with PCV2 infections experienced variable-duration viremia, with the replication and persistence of the virus being most intense in the macrophages and monocytes of the lymphoid organs and the lungs (Correa-Fiz *et al*., 2020).

2.5.4 Concurrent infections

Most PCVAD cases had concurrent infections with other swine pathogens, including PRRSV (52 per cent cases), *Mycoplasma hyopneumoniae* (36 per cent), bacterial septicemia (14 per cent) or pneumonia (7.6 per cent), and swine influenza virus (SIV) (5.4 per cent). Among these, PRRSV is the most frequent coinfection agent identified in PCVAD cases. Coinfection with PPV (up to 15 per cent in PMWS cases) have also been reported (Ellis *et al.*, 2000; Pallares *et al.*, 2002; Wellenberg *et al.*, 2004; Dorr *et al.*, 2007; Ramamoorthy and Meng, 2009; Opriessnig and Halbur, 2012).

It has been demonstrated that *M. hyopneumoniae* potentiates the severity of PCV2- induced lung and lymphoid lesions (Opriessnig *et al.*, 2004b).

It has been reported that PRRSV possibly acted synergistically with PCV2 to induce PCVAD, exaggerated immune cell depletion in pigs and increased the risk of developing PCVAD. Besides this, it has also been reported that swine pathogens such as pseudorabies virus, and Torque teno virus also may play a role in PCV2 pathogenesis (Pogranichniy *et al.*, 2002; Ramamoorthy and Meng, 2009; Ge *et al.*, 2012; Opriessnig and Halbur, 2012).

Takahashi *et al.* (2008) reported that non-suppurative polioencephalomyelitis was found in a co-infection with porcine teschovirus and PCV2 within a PCV-SD background.

2.6 PATHOGENESIS

Chang *et al*. (2006a) reported that the internalization of the virus and its presence inside of the macrophages decreased the phagocytic and microbicidal activities of the macrophages, and increased the virus' survival and spread.

In the case of PCV2-SD, the affected animals suffered from a deficiency of neutralizing antibody response and an altered cellular response (Meerts *et al.,* 2006; Fort *et al.,* 2007; Kekarainen *et al.,* 2010).

Opriessnig *et al.* (2007) reported that oronasal exposure was likely the natural route of PCV2 transmission. The virus preferentially infected lymphoid tissue in pigs and was shed through respiratory secretion, oral secretion, urine, and faeces of infected pigs.

Different cell types like epithelial cells, endothelial cells as well as macrophages showed indisputable evidence of virus replication ranging from low to very low (Hamberg *et al.,* 2007; Perez-Martin *et al.,* 2007).

Studies under *in vitro* conditions revealed that the effects of PCV2 are mostly on dendritic cells, macrophages and peripheral blood mononuclear cells. They opined that the development of reduced immune defense against other pathogens in PCV2 infection was expectable since the virus was known to impair the recognition signals by dendritic cells (DCs) (McCullough *et al.,* 2009).

Nauwynck *et al.* (2012) reported that the interaction between PCV2 and its natural hosts (domestic pig and wild boar) is a complex process that starts with the attachment of the virus and its entry into the host cell.

Segales (2012) reported that the PCV2 has a long replication cycle which may be the reason for the relatively long incubation period (around 18–25 days) to reach high viral loads in serum and tissue to develop the disease. In subclinical infection of PCV2, the interaction of the virus with the host developed a specific

adaptive response that clears the acute infection or allows virus persistence in some animals.

2.7 CLINICAL FINDINGS

In infection with PCV2, the clinical signs included weight loss, enlarged lymph nodes, jaundice, and diarrhea. Severe lymphoid depletion and histiocytic replacement in lymphoid tissue were reported to be the characteristic histological lesions (Allan *et al.*, 2000; Chae, 2012; Segales, 2012).

Reproductive failure was reported as another clinical manifestation where the aborted or stillbirth fetuses exhibited necrotizing myocarditis (Bolin *et al.*, 2001; Park *et al.*, 2005).

Respiratory distress was also manifested in some infected pigs and interstitial pneumonia and bronchiolitis with mononuclear infiltration were seen in the lungs (Harms *et al.*, 2001; Kim *et al.*, 2003).

Pigs suffering from diarrhoea due to PCV2 infection had thickened intestinal mucosa with enlarged mesenteric lymph nodes and granulomatous enteritis (Kim *et al.*, 2004; Jensen *et al.*, 2006; Opriessnig *et al.*, 2011).

Mateusen *et al.* (2007) reported that the PCV2 could replicate in embryos and that it might lead to embryonic death.

Mukherjee *et al.* (2018) reported that under field conditions, the clinical expressions of PCVAD were influenced by the genetic background of pigs on the farms and that host genetics appeared to play a role in PCV2 pathogenesis.

2.8 IMMUNOLOGY

The PMWS-affected pigs showed delayed antibody production (Bolin *et al.*, 2001; Okuda *et al.*, 2003) or produced lower antibody titres to PCV2 compared to subclinically infected ones (Hasslung *et al.*, 2005; Ladekjaer-Mikkelsen *et al.*, 2002; Rovira *et al.*, 2002).

Darwich *et al*. (2002) reported that the PCV2-infected pigs had a significantly decreased number of CD8+ and CD4+ CD8+ double-positive cell subsets compared with PCV2- negative pigs, which further indicated that PCV2 infection impaired the host immune response. The PCV2 replication was also detected in T-lymphocytes, B-lymphocytes, and monocytes that were isolated from the peripheral blood mononuclear cells (PBMCs) of infected animals. The B-cells from bronchial lymph nodes displayed a higher amount of PCV2 replication as compared to the B-cells from PBMCs (Yu *et al.*, 2007, 2009).

It has been reported by Darwich *et al.* (2003a) and Stevenson *et al.* (2006) that pigs suffering from PMWS had impaired cytokine responses.

Darwich *et al*. (2003) reported that the pigs with PCVAD had an increased level of IL-10 gene expression in the thymus and IFN-γ in tonsils as well as a decreased level of IL-2 and IL-12p40 gene expressions in the spleen; IL-4 in tonsils; and IFN-γ, IL-10, IL-12p40, and IL-4 in inguinal lymph nodes.

In a separate study, it was reported that IL-10, IL-1, and TNFα were upregulated and IL-2 and IL-4 expressions were downregulated in PCV2 infected pigs with PCVAD (Ramamoorthy and Meng, 2009; Darwich and Mateu, 2012).

Meerts *et al.* (2006) reported that the absence of neutralizing antibodies was associated with an active viral replication leading to the development of PMWS.

When exposed to the virus *in vitro*, it was observed that cultured monocytes, pulmonary macrophages, and monocyte-derived macrophages internalised PCV2. The absence of PCV2 replication in monocytic cells, despite the presence of virus-specific antigens in the cytoplasm of these afflicted cells, indicated that immune cells may not be the main site for PCV2 replication (Chang *et al.*, 2006b; Gilpin *et al.*, 2003).

Internalisation of PCV2 virus in dendritic cells demonstrated that the persistence of the virus in these cells does not affect the infectivity of the virus, cell death, and immune function of these cells which suggested that PCV2 internalization in the dendritic cells was a result of the phagocytic and endocytic

activity of the cells and not the infection of these cells by the virus. Internalisation of the virus in dendritic cells helped the virus elude the host immune system and spread throughout the body due to the dendritic cells' migratory ability (Vincent *et al.,* 2003, 2005; Kekarainen *et al.,* 2008; Steiner *et al.,* 2008).

Darwich *et al*. (2008) reported that the pigs subclinically infected by PCV2 also developed a transient elevation of IL-10 response during the peak viraemic stage.

2.9 DIAGNOSIS

Basic diagnosis of PCV2 infection relied on the detection of agreeable clinical signs, peculiar histopathological lesions, and virus presence within the lesions (Harding and Clark, 1997; Rossell *et al.,* 1999; Sorden, 2000).

Ellis *et al*. (1998) reported that antibodies to PCV2 could be detected by employing indirect immunofluorescence and immunoperoxidase test.

Allan and Ellis (2000) reported that immunohistochemistry, *in situ* hybridization, indirect immunofluorescence, PCR, PCR along with restriction fragment length polymorphism, and virus isolation were the usual methods employed for diagnosis.

2.9.1 Sample

Chung *et al.* (2005) collected nasal fluids, saliva, and joint fluids using swabs and suspended them in minimum essential medium (MEM) with a dilution factor of 5.

Segalés *et al.* (2005) in one of his studies reported that the highest PCV2 genome load was detected in tracheobronchial swabs followed by serum, tonsillar, nasal, fecal, and urinary swabs.

The PCV2 has been detected in the semen of naturally and experimentally infected boars, including seropositive animals (McIntosh *et al.,* 2006, Madson *et al.,* 2008, Schmoll *et al.,* 2008).

McIntosh *et al.* (2009) reported that the highest amount of PCV2 DNA in pooled faecal samples was detected in nursery-aged pigs in a PCVD-affected herd.

From dead pigs, organ samples such as lungs, lymph nodes, spleen, and tonsils were collected for diagnostic purpose (Anoopraj *et al.*, 2015).

2.9.2 Virus isolation

Allan *et al.* (1998) used PK-15 cell line for the isolation of PCV2 from tissue samples.

Zhu *et al.* (2007) reported that the permissiveness of PCV2 by PK-15-C1 cell clone is high when compared with PK-15 parent cells and opined that the cell line subpopulation would be useful for *in vivo* PCV2 replication and for diagnostic purposes, research, and vaccine production.

2.9.3 Serological tests

The most common diagnostic methods used to detect PCV2 antibodies in serum included indirect fluorescent assay (IFA) and immunoperoxidase monolayer assay (IPMA) (Dullac *et al.*, 1989; Allan *et al.*, 1994).

Immunohistochemical procedures based on monoclonal and polyclonal antibodies for the diagnosis of PCV2 antigen in tissues have been reported (Ellis *et al.*, 1998; Choi *et al.*, 2000).

Rodriguez *et al.* (2000) reported that the antibodies detected by indirect immunofluorescence and immunoperoxidase monolayer assay were not PCV2-specific due to the antigenic cross-reactivity between the Rep proteins encoded by PCV2 and PCV1.

Several indirect ELISA methods have been developed using the recombinant Cap protein to detect PCV2 antibodies in pig sera (Nawagitgul *et al.*, 2002). However, the indirect ELISA has some limitations including the high level of false positives due to the complex components of serum samples such as Rheumatoid factor and antibodies specific to other animal serum components.

Yin *et al.* (2010) developed an indirect ELISA for the diagnosis of PCV2, by making use of an antigenic domain (113-147AA) of ORF-2 encoded antigen expressed in E. *coli*.

Han *et al.* (2016) established a competitive ELISA (cELISA), using PCV2-specific monoclonal antibody (MAb) as the detecting antibody and virus-like particles (VLP) of PCV2 rCap protein as the coating antigen which facilitated detecting PCV2-specific antibodies from swine serum samples without PCV1 antibody interference.

2.9.4 Nucleic acid amplification methods

The polymerase chain reaction (PCR) has been used as a sensitive test for detecting a wide variety of viruses. The technique uses two oligonucleotides or primers, each of which hybridises to one strand of a DNA target, amplifying the target during the subsequent cycling steps (Mackay *et al*. 2002).

In real-time PCR, the oligonucleotide moiety that has been labelled with a fluorophore and a quencher could also be employed. The quencher reduces the fluorescence of the fluorophore through fluorescence resonance energy transfer (FRET) when the distance between two moieties is less than 100 Å. The separation of these moieties by oligonucleotide cleavage at the time of PCR increases fluorescence (Nazarenko *et al*., 2002).

Kleiboeker *et al*. (2005) reported that real-time assay was a ‘closed-tube system’, which greatly reduced the possibility of contamination and false positive findings. Additionally, it had the advantage of rapid quantification of target DNA or RNA in a single tube, increased assay specificity and the ability to simultaneously detect the multiple targets without the need of having amplification products of different sizes. The main drawback, however, was the requirement for rather expensive and sophisticated equipment, which often needed to be modified and hence revalidated before being used in a real-time quantitative format.

Real-time PCR technology combined PCR and the use of fluorescent reporter molecules to detect amplification products throughout each PCR cycle. The

different real-time PCR chemistries have been classified into double-stranded DNA intercalating molecules (SYBR Green I and EvaGreen) and fluorophore-labeled oligonucleotides (Scorpions, LUX probes, TaqMan probes, MGB-TaqMan probes, Molecular Beacons *etc*.) (Navarro *et al*., 2015).

2.9.4.1 ***Detection of PCV2 by different PCR techniques***

2.9.4.1.1 Detection of PCV2 by conventional PCR

Ellis *et al.* (1999) reported the use of PCR to detect PCV2 without cross-reaction with PCV1. The PCR targeted a 481 bp fragment of the ORF 2 of the virus.

Calsamiglia *et al.* (2002) reported that the most sensitive method for the detection of PCV2 from superficial inguinal lymph nodes was PCR.

In Meghalaya, Mukherjee *et al.* (2018) employed PCR to test 190 tissue samples, collected during necropsy of pigs suspected to have died of PCV-2 infection and found that 36 (18.94 per cent) were positive.

2.9.4.1.2 TaqMan probe based real time PCR

Olvera *et al.* (2004) reported the standardization of a TaqMan real-time PCR, targeting the ORF2 of PCV2, for detection and quantitation of viral load in the tissues of pigs affected with PMWS and PDNS. They observed that the tissues of pigs diagnosed with PMWS had a higher virus load when compared to those of animals with PDNS.

Brunborg *et al.* (2004) established a TaqMan-based real-time PCR for the quantitation of PCV2 in tissue and plasma/serum samples.

Zhao *et al.* (2010) reported a sensitive, specific TaqMan-based real-time PCR to detect and quantify PCV2 by designing and synthesizing specific primers and probes in the ORF2 of the viral genome. They also reported that the assay was more sensitive, had less chance of contamination, and could be quickly performed and had the highest detection rate compared to conventional PCR for the detection of the PCV2.

Henriques *et al.* (2018) reported the development and validation of quantitative PCR (qPCR) for the detection of PCV2 in swine samples, using primers and a TaqMan probe directed to a conserved region of the replicase encoding gene.

2.9.4.1.3 SYBR Green real-time PCR

Yang *et al.* (2007) reported that for rapid and simple clinical diagnosis of PCV2, SYBR Green I-based real-time PCR could be used with high sensitivity and specificity as it showed no cross-reaction with PCV1.

McIntosh *et al*. (2009) developed and validated a novel SYBR green real-time polymerase chain reaction (PCR) assay targeting ORF1 (rep) exploiting oligonucleotide primer binding sites conserved across 244 characterized PCV2 genomes and investigated the effects of diagnostic specimen choice on its performance.

2.9.4.1.4 Multiplex PCR (mPCR)

Giammarioli *et al.* (2008) developed a novel hot-start mPCR for the simultaneous detection of five swine viruses like CSF virus (CSFV), African swine fever virus (ASFV), PCV2, PPV, and PRRSV and confirmed that their sensitivity in correspondence with the single PCR for detection of each of the five targets was identical for CSFV, PCV2 and PPV, one log lower for PRRSV and two logs lower for ASFV.

Jiang *et al.* (2010) developed mPCR for four viruses causing reproductive and respiratory failure in pigs, PCV2, PPV, CSFV, and PRRSV, which helped in the detection of co-infections with these viruses. This method was a rapid, sensitive, cost-effective diagnostic method for the routine surveillance of viral infections in pigs.

Xu *et al.* (2012) developed uniplex RT-PCR and multiplex RT-PCR to detect six viruses of pigs, which comprised PRRSV, CSFV, PCV2, PPV, porcine pseudorabies virus, and Japanese encephalitis virus. They found multiplex RT-PCR

to be more sensitive and specific than uniplex RT-PCR and could be used efficiently to detect multiple infections.

2.9.4.1.5 Nested PCR

Kiatipattanasakul-Banlunara *et al.* (2002) conducted a retrospective study for the detection of PCV2 DNA in 16 cases of swine PMWS in Thailand employing nested PCR.

Lyoo *et al.* (2008) reported that for the detection of PCV2 and to differentiate PCV2a and PCV2b in field samples nested PCR could be used.

Kim *et al.* (2017) reported that there are high levels of co-incidence of porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) in porcine tissue. In this study, a duplex nested reverse transcriptase polymerase chain reaction (RT-PCR) method that targeted the genomic RNA of type 2 PRRSV and the mRNA of PCV2 in infected tissues was developed. The method amplified discriminative bands of 347 bp and 265 bp specific for type 2 PRRSV and PCV2, respectively.

2.9.4.1.6 Loop-Mediated Isothermal Amplification (LAMP)

Chen *et al.* (2008) developed a loop-mediated isothermal amplification (LAMP) method for rapid detection of PCV2. The LAMP products could be determined by agarose gel electrophoresis or by adding SYBR Green I dye. The amplification of LAMP could be obtained at 63°C for 60 min. The detection limit was nearly 1 copy of DNA plasmid which was more sensitive than PCR. There was no cross-reaction with PCV1, porcine pseudorabies virus (PRV), and porcine parvovirus (PPV) under the same conditions. They opined that LAMP was a useful rapid detection method with high sensitivity and specificity for PCV2.

2.9.5 Restriction Fragment Length Polymorphism (RFLP) and PCR-based RFLP

Ciacci-Zanella *et al*. (2003) reported that swine organ samples were analysed by PCR to amplify a sequence of PCV2 DNA present in those tissues.

This test amplifies sequences of PCV2 and also of PCV1. PCR amplified products were analysed by RFLP using restriction enzymes. This test identified PCV1, PCV2A, B, C, D, or E, using the enzymes *KpnI* or *HinfI*. Thus, it was possible to identify PCV2 and differentiate it from PCV1.

2.9.6 Phylogenetic analysis

The Cap gene is a reliable phylogenetic and epidemiological marker for PCV2 strains since it was able to reconstruct the same tree as the whole viral genome. This is because the Cap gene is under less selective pressure compared with the Rep gene, accumulating much more variability, and it probably weighs more in the phylogenetic tree reconstructions and also it was not affected by recombination, in contrast to the Rep gene (Olvera *et al*., 2007)

De Castro *et al.* (2007) reported that both PCV2a and PCV2b have been detected in Brazil. De Castro *et al.* (2012) sequenced the capsid protein gene (ORF2) of the Brazilian isolates of PCV2 and subjected the sequences to phylogenetic analysis. They could observe that among the isolates studied, PCV2b isolates predominates and only one isolate was detected as PCV2a.

The PCV2a and PCV2b had a worldwide distribution with PCV2b being the predominant genotype detected since 2003. PCV2c has been reported only in Denmark from the archived material (Dupont *et al.,* 2008), while PCV2d isolates have been reported from China which fit into the existing definition of genotype (Guo *et al.,* 2010).

The predominance of type 2b in herds with PCVAD has been reported from several countries in Europe and in North America whereas before PCVAD reporting PCV2a has been recovered in Canada, in healthy animals of Switzerland, and from non-PCVAD affected farms of USA (Segales *et al.,* 2008, Cortey *et al.,* 2011).

Since 2015, the new genotype PCV2e was circulating in the United States. Its ORF2 sequences had 12 or 15 additional nucleotides compared to PCV2a-PCV2d (Harmon *et al.,* 2015, Davies *et al.,* 2016).

With the phylogenetic analysis based on the Cap gene and complete genome, PCV2 could be classified into four genotypes: PCV2a (earlier group II) with five subtypes or clusters (2A, 2B, 2C, 2D, and 2E), PCV2b (earlier group I) with three clusters (1A, 1B, and 1C), PCV2c and more recently reported PCV2d from China (Segales *et al.*, 2008, Guo *et al.*, 2010).

Franzo *et al.* (2015) estimated the nucleotide substitution rate at 1.4×10^{-3} to 7.8×10^{-4} per site per year, with a higher rate for the genotype PCV2a compared to PCV2b and PCV2d, and a slightly higher rate for the ORF2 compared to ORF1.

Franzo and Segales (2018) proposed a new genotyping methodology based on a phylogeny-grounded genotype definition including three requirements: a maximum intra-genotype p-distance of the ORF2 sequence of 13 per cent, bootstrap support at the corresponding internal node of more than 70 per cent, and at least fifteen available sequences. This classification scheme allowed the definition of eight PCV2 genotypes, including the previously described genotypes PCV2a to PCV2f and the two new genotypes PCV2g and PCV2h.

Bhattacharjee *et al*. (2021) reported that in India, the genotypes of PCV2 prevalent were 2a, 2b, 2d, 2f, and 2h, and that the clusters prevalent were 2, 3, 10, 11, 13, and 18.

2.10 PREVENTION AND CONTROL

According to MacLachlan and Dubovi (2011), general management strategies to reduce circovirus infections along with those driven on by additional, presumably "secondary," pathogens that may act as triggers for improved PCV2 replication were among the many methods that should be implemented to control pig circovirus associated disorders. Disinfection was essential to prevent the spread of the virus between groups, also nutrition and hygiene should be good.

Comprehensive measures include “all-in” and “all-out” protocols, disinfection, minimizing animal contact, restricting batch mixing and cross-fostering, and isolating or euthanizing sick pig. The maintenance of the

environmental factors such as appropriate temperature, air-flow, and space within pens; and in addition, appropriate use of anti-parasitic treatments and vaccination (Madec *et al*., 2000), control of co-infections (Opriessnig *et al*., 2003), monitoring the breeding and semen quality (Madson *et al*., 2009), and improvement of herd nutrition (Bassaganya-Riera *et al*., 2003) were also important in prevention and control of the infection.

2.10.1 Vaccination

Fort *et al*. (2008) reported that the double dose of the subunit vaccine containing PCV2 capsid protein was proved to prevent viremia in experimentally infected animals.

Four PCV2 vaccines were commercially available in most countries: one sow vaccine (Circovac R, Merial) and three piglet vaccines (Ingelvac R Circo FLEX, Boehringer Ingelheim and Porcilis R PCV/Circumvent R PCV). All vaccines were inactivated and based on the PCV2a genotype. With the commercialization and use of the available PCV2a vaccine, it was speculated if the efficacy of vaccine based on PCV2a might risk the fact that most infections are caused by PCV2b strain but studies revealed that it seems not the case (Fort *et al.*, 2008).

Madson and Opriessnig (2011) opined that the shedding of PCV2 in semen and the prevalence of intrauterine-infected piglets can both be greatly reduced by PCV2 vaccination well ahead of expected PCV2 exposure.

Even though no overt clinical signs were observed, different field evidence indicated that PCV2 vaccination could improve productive parameters (average daily gain, percentage of runts, body condition, and carcass weight) in PCV2 subclinical infection scenarios (Young *et al*., 2011).

Beach and Meng (2012) reported that after the introduction of the PCV2 vaccine, a change in the impact of PCV2 on pig production was observed as viremia was controlled efficiently.

According to Mukherjee *et al.* (2018) it could be assumed that extensive use of PCV2 vaccines has changed the epidemiology of PCVADs including the reduction of losses related to PMWS in many countries but unfortunately, the PCV2 vaccination program is yet to start in some parts and is confined in some parts of Indian subcontinent which increases the risk factor of PMWS in small herds and pig industries in this country.

Materials and methods

3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals

All the chemicals employed in this study were of Analar or Molecular biology grade. Ethanol, isopropanol, agarose, tris, calcium chloride, and glycerol were procured from Merck (Germany). Ethidium bromide and ethylene diamine tetra acetic acid (EDTA) were purchased from Sisco Research Laboratories (SRL) (India). Chemicals for molecular biology work were purchased from reputed firms such as Thermo Scientific (USA), Invitrogen (USA), Applied Biosystems (USA), TakaRa (Japan), and Merck (Germany).

3.1.2 Glassware and labware

The glassware used in this study for various purposes was procured from Borosil (India) and labware from Tarsons (India). The labware used in DNA extractions and PCR works was certified DNase, RNase, and protease-free. Filter tips were used for all pipetting applications.

3.1.3 Buffers and reagents

The compositions of all the buffers and reagents used in this study have been mentioned in the appendix.

3.1.4 Equipment

Mastercycler Nexus Gradient thermal cycler (Eppendorf, USA), Step One Plus Real-time PCR machine (Applied Biosystems, Singapore), ultra-low temperature freezer (-80°C) (New Brunswick Scientific, England), refrigerated centrifuge (Eppendorf, Germany), gel documentation system (Syngene, U. K.), Qubit 4 fluorometer (Thermo Scientific, USA), Spinwin microfuge (Bangalore Genei, India), vortex mixer (REMI equipments, India), laminar airflow cabinet (Labline, India), shaker incubator (Labline, India), dry bath (Techne, UK), hot air oven (Rotek, India), bacteriological incubator (Labline, India), microwave oven (IFB appliances, China), submarine horizontal electrophoresis system (Biotech,

India), electronic weighing balance (Sartorius, USA), and micropipettes (Finpipette, Finland and Eppendorf, Germany) were used.

3.1.5 Bacterial culture medium

Luria Bertani (LB) agar and LB broth, used for bacterial culture for cloning experiments were from Himedia (Mumbai, India) and the method of preparation is given in the appendix.

3.1.6 Enzymes

Enzymes used in the study were purchased from Thermo Scientific (USA).

3.1.7 Oligonucleotide primers and probes

Oligonucleotide primers and TaqMan probes were custom synthesized from Sigma (India/USA). The details of the primers and probes used in the study are given in table 2.

3.1.8 Cloning vector

The pJET 1.2/blunt vector (Thermo Scientific, USA) was used for cloning the gel-purified PCR amplicon (Plate 1).

3.1.9 Bacterial (competent) cells

DH5α strain of *Escherichia coli* (*E. coli*) was employed for the preparation of competent cells required for transformation.

3.1.10 Molecular biology and other kits

a) DNeasy Blood & Tissue Kit (QIAGEN, Germany)

b) GeneJET Gel Extraction Kit (Thermo Scientific, USA)

c) CloneJET PCR cloning kit (Thermo Scientific, USA)

d) Qubit™ (1X dsDNA HS Assay and dsDNA BR Assay) kits (Thermo Scientific, USA)

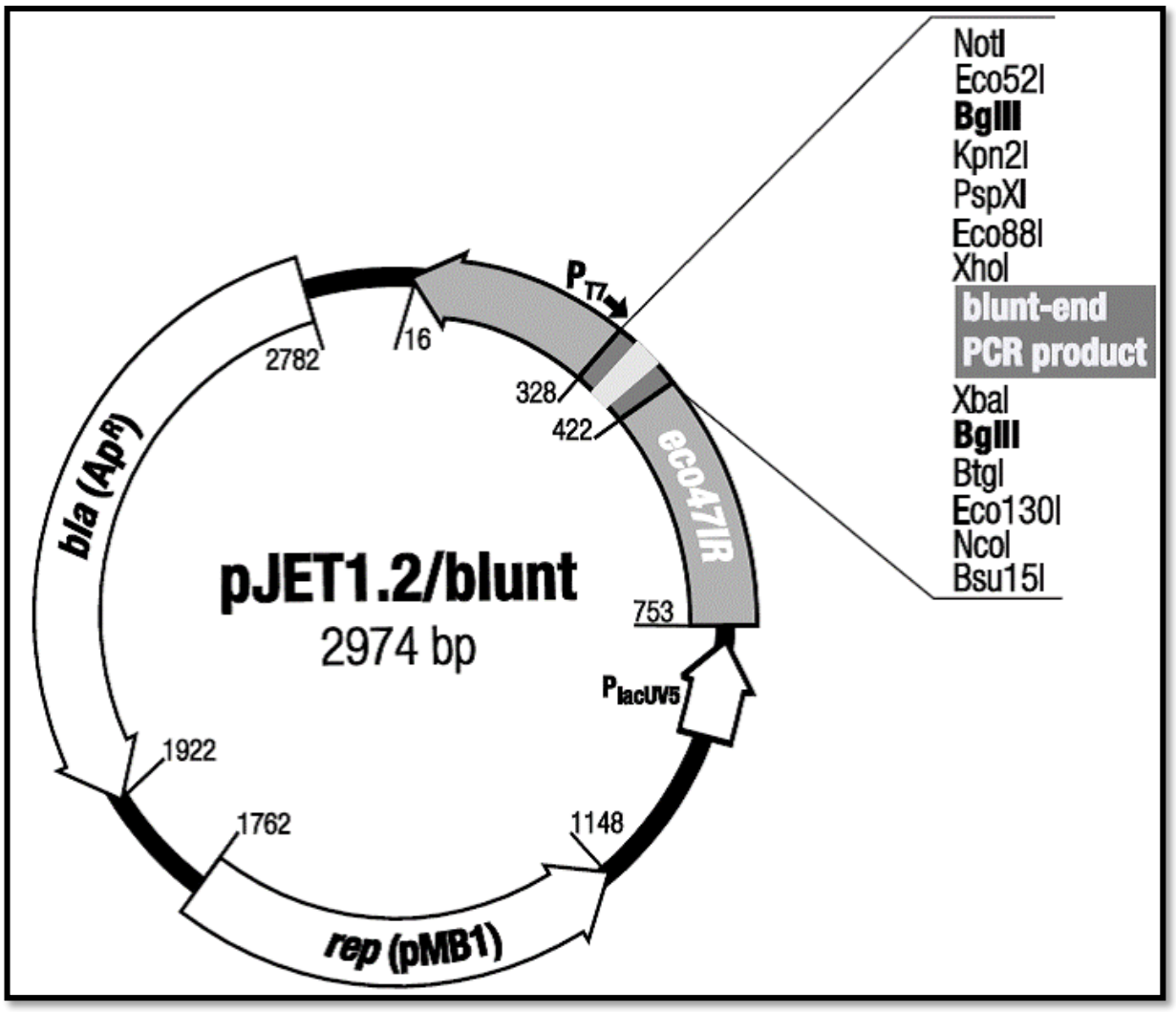


Plate 1. Map of cloning vector (pJET1.2/blunt vector)

3.1.11 Samples

For detection of PCV2, tonsil, mesenteric lymph node, lung, liver, and spleen were collected from pigs that have died of respiratory illness in Wayanad, Kozhikode, Kannur and Thrissur districts of Kerala. A total of 62 tissue samples were used for the study. The samples were collected from 2021 to August 2022. The details of the samples collected are given in Table 1.

3.2 METHODS

3.2.1 Collection of samples

The tissue samples were collected in sterile screw-capped polypropylene vials using sterile scissors and forceps and were processed immediately or stored at -80°C after proper labelling to prevent DNA degradation till further processing.

Table 1. Details of the tissue samples collected for the detection of PCV2

Sl. No.	Register/Sample Number	District	Clinical symptoms and post-mortem lesions observed
1	16/MIB/2021	Wayanad	Diarrhoea, anorexia, weight loss, enteritis.
2	17/MIB/2021	Wayanad	Diarrhoea, anorexia, weight loss, enteritis.
3	33/MIB/2021	Thrissur	Pneumonic lungs, mesenteric lymphadenopathy, ulcers in caecal mucosa, diphtheritic membrane in stomach and intestine.
4	34/MIB/2021	Thrissur	Consolidation in lungs, diphtheritic membrane in stomach and intestine, ulcers in caecal mucosa, congested serous mucosa of the stomach, mesenteric lymphadenopathy
5	47/MIB/2021	Kozhikode	Bronchopneumonia, hydropericardium, pyelonephritis, hydro pericardial fluid.
6	78/MIB/2021	Wayanad	Dyspnoea, hydrothorax, pneumonia, hydroperitoneum, enteric necrotic hepatitis.
7	118/MIB/2021	Kozhikode	Ascites, pulmonary oedema, hydrothorax, hydropericardium, myocarditis, and ulcers in the rectum.

8	122/MIB/2021	Wayanad	Pale liver, pale kidney, pale spleen, pulmonary emphysema
9	123/MIB/2021	Wayanad	Ascites, hydrothorax, hydropericardium, pulmonary oedema.
10	124/MIB/2021	Wayanad	Ascites, hydrothorax, hydropericardium, pulmonary oedema.
11	127/MIB/2021	Wayanad	Congestion of intestine, mesenteric blood vessels.
12	128/MIB/2021	Wayanad	Congestion of intestine, mesenteric blood vessels, and gastrointestinal junction.
13	129/MIB/2021	Wayanad	Congestion of the intestine, mesenteric blood vessels, congestion of the liver.
14	132/MIB/2021	Wayanad	Diarrhoea, vomiting, hydropericardium.
15	133/MIB/2021	Wayanad	Diarrhoea, vomiting, hydropericardium.
16	146/MIB/2021	Wayanad	Pneumonia, enlarged lymph nodes with haemorrhage, ulcers in the caecum and colon.
17	168/MIB/2021	Kozhikode	Loss of body weight, diarrhoea after weaning, fibrinous pleuritis, hydropericardium, lymph node enlarged, and haemorrhagic.
18	181/MIB/2021	Wayanad	Diarrhoea, inappetence, intestinal congestion, haemorrhagic lymph node, pinpoint ulcers in the caecum and colon.
19	248/MIB/2021	Wayanad	Fibrinous deposits in the peritoneum, pleura, pericardium, necrotic foci in the liver, and hydropericardium.
20	256/MIB/2021	Thrissur	Gastroenteritis, epicardial haemorrhage, meningitis, cerebral oedema.
21	260/MIB/2021	Wayanad	Gastroenteritis, lymphadenopathy, pulmonary emphysema, pneumonia, fibrous deposits in the peritoneum, nephritis.
22	261/MIB/2021	Wayanad	Haemorrhagic gastroenteritis, haemorrhagic mesenteric lymph node, fibrous deposits in the peritoneum, hydrothorax, hydropericardium.
23	269/MIB/2021	Wayanad	Laboured breathing, bronchopneumonia.
24	272/MIB/2021	Wayanad	Suppurative bronchopneumonia, cranioventral consolidation, and pus in the liver.
25	273/MIB/2021	Wayanad	Pulmonary oedema and congestion

26	312/MIB/2021	Wayanad	Lobular pneumonia, diphtheritic membrane formation in the intestine.
27	316/MIB/2021	Wayanad	Laboured breathing, bronchopneumonia.
28	353/MIB/2021	Wayanad	Mucoid exudate in bronchi and trachea, bronchopneumonia, and catarrhal enteritis.
29	370/MIB/2021	Wayanad	Diarrhoea, anorexia, weight loss, enteritis, mesenteric lymph node enlargement, mesenteric blood vessel congestion.
30	371/MIB/2021	Wayanad	Weight loss, anorexia, diarrhoea, enteritis, diphtheritic membrane in the stomach.
31	384/MIB/2021	Wayanad	Icterus, hepatitis, splenomegaly, diffuse consolidation of lobes, ascites, hydropericardium.
32	394/MIB/2021	Wayanad	Suppurative pneumonia, pus in the liver, lymph nodes enlarged, anaemia.
33	399/MIB/2021	Wayanad	Diffused petechial haemorrhages on the skin, subcutaneous tissues on the thigh and lateral abdomen, haemorrhagic mesentery, and petechial haemorrhages all over the gastric intestinal mucosa.
34	427/MIB/2021	Wayanad	Fibrinous pericarditis, pulmonary haemorrhages, hemorrhagic lymph node, endocardial haemorrhage, valvular haemorrhage, enteritis.
35	431/MIB/2021	Wayanad	Gastritis, tonsillar necrosis, non-collapsed lungs, catarrhal enteritis, diphtheritic membrane in the ileum, caecal and colonic ulcers.
36	432/MIB/2021	Wayanad	Weight loss, inappetence, non-collapsed, oedematous lungs, frothy and mucoid exudate in the trachea, enteritis.
37	437/MIB/2021	Wayanad	Emaciated carcass, dehydration, enteritis, pneumonia.
38	454/MIB/2021	Wayanad	Red discoloration of the skin, pulmonary congestion.
39	35/MIB/2022	Wayanad	Blood-tinged fluid in the abdomen and thoracic cavity, ecchymotic focal haemorrhages on skin.
40	40/MIB/2022	Kannur	Caseous pus material in scrotum, small ulceration in ileum, congested mesenteric lymph node.

41	42/MIB/2022	Wayanad	Diarrhoea, haemorrhagic mesenteric lymph nodes, intestinal mucosal haemorrhage.
42	45/MIB/2022	Wayanad	Consolidated lungs, fibrinous pericarditis, hydrothorax, hydropericardium, and disseminated degenerative changes in the liver.
43	58/MIB/2022	Wayanad	Haemorrhagic skin lesion.
44	62/MIB/2022	Wayanad	Congested mesenteric lymph node, catarrhal enteritis, endocardial haemorrhage.
45	71/MIB/2022	Wayanad	Turkey egg appearance of kidney, haemorrhagic lymph nodes, infarct in lungs.
46	72/MIB/2022	Wayanad	Necrotic fibrinous pneumonic change in lungs, splenic nodule, congested lymph node.
47	73/MIB/2022	Wayanad	Pneumonic changes in lung, gastritis, sudden death.
48	88/MIB/2022	Wayanad	Enteritis
49	89/MIB/2022	Wayanad	Enteritis
50	103/MIB/2022	Wayanad	Diarrhoea, sudden death, putrefied carcass.
51	121/MIB/2022	Kozhikode	Nephropathy, dermatitis, anaemia, pneumonia.
52	148/MIB/2022	Wayanad	Petechial haemorrhage on the epicardium, lung and heart adhesion, pleuritis.
53	169/MIB/2022	Wayanad	Pneumonia, haemorrhagic lymph node, epicardial haemorrhage.
54	186/MIB/2022	Wayanad	Vomiting, diarrhoea, haemorrhagic enteritis.
55	188/MIB/2022	Wayanad	Dermatitis, spleen infarcts, lung oedema.
56	189/MIB/2022	Kozhikode	Dermatitis, nephrosis, oedematous right lung lobe, congested mesenteric lymph node
57	192/MIB/2022	Wayanad	Dermatitis
58	194/MIB/2022	Wayanad	Pleuritis, pericarditis.
59	242/MIB/2022	Wayanad	Dermatitis, lung oedema.
60	248/MIB/2022	Wayanad	Pneumonia
61	257/MIB/2022	Wayanad	Nephropathy, hepatopathy, pneumonia.
62	294/MIB/2022	Wayanad	Pneumonia, skin lesions, haemorrhagic enteritis.

3.2.2 Molecular confirmation of PCV2 in clinical Samples

3.2.2.1 Detection of PCV2 by conventional PCR

3.2.2.1.1 DNA extraction from tissue samples

Total DNA was isolated from the tissue samples using DNeasy Blood & Tissue Kit (QIAGEN, Germany), as per the manufacturer's protocol. Briefly, 10 mg of tissues were cut up into small pieces and kept in a 1.5 ml microcentrifuge tube for incubation at 56℃ after adding 180 µL Buffer ATL and 20 µL Proteinase K with occasional vortexing until tissue lysed completely. In the case of blood, 20 µL Proteinase K was added to 50 µL of blood and made the volume 220 µL by adding PBS. After adding 200 µL Buffer AL it was incubated at 56℃ for 10 min. Subsequently, 200 µL ethanol was added and vortexed thoroughly. Into the completely lysed tissue suspension 200 µL Buffer AL and ethanol were added and mixed thoroughly by vortexing. Pipetted out the mixture into DNeasy Mini spin column placed in 2 mL collection tube and centrifuged at 8000 rpm for 1 minute. Flow-through was discarded and the spin column was placed in a new collection tube and centrifuged at 8000 rpm for 1 minute after adding 500 µL AW1. Discarded the flow-through and centrifuged again at 14000 rpm for 3 minutes after placing it in a new collection tube and adding 500 µL AW2. Finally, the spin column was placed in a 1.5 mL microcentrifuge tube and centrifuged at 8000 rpm for 1 minute after adding 200 µL Buffer AE and stored at -20℃.

3.2.2.1.2 Detection of PCV2 by conventional PCR

The presence of the PCV2 genome in the clinical samples was detected by PCR using primer specific to ORF2 as described by Ellis *et al.* (1999) with slight modifications. Details of the primers used for the detection of PCV2 are given in table 2. The 25 µL reaction mix consisted of 12.5 µL of 2X Emerald Amp GT PCR Mastermix containing Taq polymerase (TaKaRa), 1 µL each of 10 pmol PCV2 forward and reverse primers, 2 µL of DNA, and rest NFW. The cycling conditions for amplification of PCV2 were 95℃ for 2 min (initial denaturation), 34 cycles of 94℃ for 30 seconds (denaturation), 55℃ for 45 seconds (annealing), and 72℃ for

45 seconds (polymerization) followed by single cycle at 72℃ for 5 min (final extension). No template control was included in each run.

Table 2. Details of PCR primers and probes used for detection of PCV2

Set	Primer name	Sequence 5'-3'	Target region/ gene	Annealing temp with time	Product size (bp)	Reference
1	PCV2 FP	CGGATATTGTAGTCC TGGTCG	ORF 2	55℃, 1 min	481	Ellis *et al.* (1999)
	PCV2 RP	ACTGTCAAGGCTACC ACAGTC				
2	PCV2 1F	CGAGAAAGCSAAAG GAACWGA	332–353	53℃, 45 sec	661	Anoopraj *et al.* (2015)
	PCV2 1R	GGGGGYAGGGTGAC SAA	991–975			
3	PCV2 2F	AAGGGAGCCAGTTC GTCACC	964–983	62℃, 40 sec	765	
	PCV2 2R	CGCCCCCGCAGCCAT CTT	1729–1712			
4	PCV2 3F	AGGAGGGCGTTCTG ACTG	1570–1587	60℃, 40 sec	676	
	PCV2 3R	GACTCCCGCTCTCCA ACAAG	477–458			
5	PCV2 RT Ker F	TCCASTGCTGTTATT CTA	ORF2	57℃, 30sec	81	This study
	PCV2 PKD Taq-RP	GGGAGGAGTAGTTT ACATA				
	PCV2 Ker Probe	6FAM-TGTAMCAAAGGCCA MWGCCCTVAC-BHQ1				
6	PCV2 PKD Taq-FP	TCCACTGCTGTTATT CTA	ORF2	57℃, 30sec	81	This study

	PCV2 PKD Taq-RP	GGGAGGAGTAGTTTACATA				
	PCV2 PKD TaqMan probe	6FAM-TGTAACAAAGGCCAATGCCCTAAC-BHQ1				

3.2.2.1.3 Visualization of the PCR product

Agarose gel of 1.25 per cent concentration was prepared with 0.5X TBE buffer. Agarose was weighed and dissolved in 0.5X (Tris-Borate EDTA) TBE buffer by heating and was cooled to 50°C. Ethidium bromide was added to a final concentration of 0.5 µg/mL to the melted agarose. The gel was poured into the gel tray after placing the comb. After the gel has set, the comb was removed and the gel was placed in the electrophoresis apparatus. The 12.5µL of the PCR product was loaded into wells. Five microlitres of 100 bp DNA ladder (Thermo Scientific, USA) were loaded in one of the wells as the molecular weight marker. Electrophoresis was carried out at 100V until bromothymol blue dye has migrated to more than two-thirds of the gel. The gel was visualized to identify the size of the product and a photograph was taken in a gel documentation system under UV illumination.

3.2.3 Genotyping of PCV2

Eleven representative positive samples were subjected to PCR for amplification of the complete genome of the virus using published primers (Anoopraj *et al*., 2015) that amplified three overlapping fragments of size 661, 765, and 676 bp (Table 2, Set 2 to 4). After the run, visualization of the PCR product was carried out as described in step 3.2.2.1.3.

3.2.3.1 Gel purification of the PCR product

The 661/765/676 bp amplicons generated by PCR were purified from the gel by using GeneJET Gel Extraction Kit (Thermo Scientific, USA) as per the manufacturer's protocol. The DNA fragment was visualized under a UV

transilluminator and was excised from the agarose gel using a sterilized clean and sharp scalpel or razor blade as close to the DNA as possible to minimize the gel volume. The gel slice was placed into a pre-weighed 1.5 mL tube and the weight of the gel slice was recorded. Added 1:1 volume of Binding buffer to the slice and the gel mixture was incubated at 50-60℃ for 10 minutes or until the gel slice is completely dissolved. The tube was mixed thoroughly by inverting every few minutes to facilitate the melting process. After ensuring that the gel is completely dissolved, the gel mixture was briefly vortexed before loading on the GeneJET purification column. Transferred up to 800 µL of the solubilized gel solution to the column. Centrifuged for 1 minute. The flow-through was discarded and the column was placed back into the same collection tube. Added 100 µL of Binding buffer to the purification column, centrifuged for 1 minute and after discarding the flow-through, the column was placed back into the same column. Added 700 µL of Wash buffer which was diluted with ethanol to the column and centrifuged for 1 minute. The flow-through was discarded and the column was placed back into the same collection tube. Centrifuged the empty purification column for an additional 1 minute to completely remove residual wash buffer. Transferred the GeneJET purification column into a clean 1.5 mL microcentrifuge tube and added 50 µL of Elution buffer to the centre of the purification column membrane. Centrifuged for 1 minute and discarded the GeneJET purification column and stored the purified DNA at -20℃.

3.2.3.2 Sequencing of the PCR products

Sequencing of samples 33/MIB/2021, 47/MIB/2021, 78/MIB/2021, 118/MIB/2021, 122/MIB/2021, 127/MIB/2021, 128/MIB/2021, 146/MIB/2021, 248/MIB/2021, 256/MIB/2021 and 261/MIB/2021 were carried out at M/s AgriGenome Labs Private Limited, Kakkanad, Cochin, Kerala.

3.2.3.3 Analysis of nucleotide sequences

The chromatograms received after sequencing were analysed using Chromas Lite v2.01 software (http://www.technelysium.com.au) and sequence

reading errors if any were edited. BLAST was performed with the sequences within the non-redundant nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the presence of the gene-specific to PCV2. The complete genomes assembled from the three fragments using Emboss Merger (https://www.bioinformatics.nl/cgi-bin/emboss/merger). The sequences were submitted to GeneBank (https:/www.ncbi.nlm.nih.gov/genbank/) and the accession numbers were obtained.

3.2.3.4 Phylogenetic analysis

Sequences of PCV2 reported from other countries and also India were obtained from GenBank and included in the sequence comparison. Phylogenetic and molecular evolutionary analyses of the sequences were done using MEGA X software (Kumar *et al*., 2018). The sequences obtained during this study and those downloaded from GenBank were aligned using the Clustal W program of MEGA X software, followed by trimming of the same to match sequence lengths obtained in this study. The evolutionary history was inferred by using the Maximum Likelihood method. The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of sequences analysed. Three phylogenetic trees were generated, a tree from the partial ORF2 sequences, one from the complete ORF2 and the third one from the complete genome. The evolutionary distances used to infer the phylogenetic trees were computed using the Kimura 2 Parameter model for partial and complete ORF2 sequences and Tamura-Nei Parameter model for complete genome as they had the lowest Bayesian Information Criterion scores (BIC). A sequence of PCV1 was also included in the analysis to serve as an outgroup. The details of sequences employed for the phylogenetic analysis are given in table 3.

Table 3. Details of isolates of PCV2 downloaded from GenBank for phylogenetic analysis

Sl. No.	Accession number	Country	Year of collection or year submitted to NCBI
1	MT828905*	India (Kerala)	2021
2	MW268739*	India (Kerala)	2021
3	MW268738*	India (Kerala)	2021
4	KX009480*	India (Mizoram)	2016
5	KX009481*	India (Mizoram)	2016
6	LC004752*	India (Meghalaya)	2014
7	GU808525*	India (Uttar Pradesh)	2010
8	KJ729075*	India (Uttar Pradesh)	2015
9	KX009482*	India (Mizoram)	2016
10	KJ729074*	India (Uttar Pradesh)	2015
11	KJ729072*	India (Uttar Pradesh)	2015
12	KJ729073*	India (Uttar Pradesh)	2015
13	LC004732*	India (Meghalaya)	2015
14	LC004734*	India (Meghalaya)	2014
15	JX948768*	China	2013
16	KX960931*	China	2017
17	JF690919*	Malaysia	2011
18	JF690920*	Malaysia	2011
19	KU317473*	China	2016
20	KM235959*	China	2016
21	GQ449669*	China	2011
22	KC514972*	China	2015
23	MF314280*	Thailand	2017
24	MF314275*	Thailand	2017
25	MF314270*	Thailand	2017
26	MF314261*	Thailand	2017
27	MF314260*	Thailand	2015
28	KC823058*	China	2013
29	MF589543*	China	2018
30	JF317582*	South Korea	2012
31	JF317589*	South Korea	2012
32	KC515012*	China	2015
33	KM042394*	Vietnam	2016
34	JX099782*	Vietnam	2014
35	KM042395*	Vietnam	2016
36	KM042396*	Vietnam	2016
37	LC004736*	India (Northeast states)	2014
38	LC004738*	India (Northeast states)	2014
39	LC004739*	India (Northeast states)	2014
40	LC004745*	India (Northeast states)	2014
41	LC004746*	India (Northeast states)	2014
42	LC004748*	India (Northeast states)	2014

43	LC004749*	India (Northeast states)	2014
44	LC004750*	India (Northeast states)	2014
45	LC004751*	India (Northeast states)	2014
46	LC004753*	India (Northeast states)	2014
47	LC008134*	India (Northeast states)	2014
48	LC008135*	India (Northeast states)	2014
49	LC008137*	India (Northeast states)	2014
50	LC008141*	India (Northeast states)	2014
51	MF326365*	China	2018
52	MF326369*	China	2018
53	MF326372*	China	2018
54	MF589534*	China	2018
55	MF589526*	China	2018
56	KC514996*	China	2015
57	MF589538*	China	2018
58	MF589523*	China	2018
59	MF589524*	China	2018
60	JF690922*	Malaysia	2011
61	JF690923*	Malaysia	2011
62	MK770354*	India	2019
50	LC008141*	India	2014
51	MF326365*	China	2018
52	MF326369*	China	2018
53	MF326372*	China	2018
54	MF589534*	China	2018
55	MF589526*	China	2018
56	KC514996*	China	2015
57	MF589538*	China	2018
58	MF589523*	China	2018
59	MF589524*	China	2018
60	JF690922*	Malaysia	2011
61	JF690923*	Malaysia	2011
62	LC010925	India (Meghalaya)	2014
63	KJ415567	India	2015
64	KJ415568	India	2015
65	KX579945	India (Kerala)	2017
66	MW477471	India (Kerala)	2021
67	MW477472	India (Kerala)	2021
68	MW477473	India (Kerala)	2021
69	MW477474	India (Kerala)	2021
70	MW477475	India (Kerala)	2021
71	MW477476	India (Kerala)	2021
72	MW477477	India (Kerala)	2021
73	MW051794	India (Kerala)	2021

74	MW051795	India (Kerala)	2021
75	MW125684*	India (Tamil Nadu)	2021
76	MZ197169*	India (Tamil Nadu)	2021
77	MW125680*	India (Tamil Nadu)	2021
78	MW790264*	India (Tamil Nadu)	2021
79	MW790260*	India (Tamil Nadu)	2021
80	MZ254671*	India (Tamil Nadu)	2021
81	MW125678*	India (Tamil Nadu)	2021
82	MK770354*	India	2019

* Isolates used for complete ORF2 sequence phylogenetic analysis

3.2.4 TaqMan real-time PCR

3.2.4.1 Primer designing for TaqMan real-time PCR

The sequences obtained during the study and all the sequences of Indian isolates available in GenBank were aligned using ClustalW program of MEGA X software (Kumar *et al*., 2018). Primers and probes were designed from the conserved ORF2 region of the aligned sequences using the online OligaArchitect software (www.sigmaaldrich.com). Initially a set of primers and a probe was designed to detect all the genotypes of PCV2 prevalent in Kerala. Later a forward primer and probe were designed to detect the predominant genotype of PCV2 in Kerala (2d genotype). The details of the primer and probe sequences are given in table 2 (Set 5 and 6).

3.2.4.2 Standardization of TaqMan real-time PCR for detection of PCV2

3.2.4.2.1 Standardization using real-time TaqMan PCR primer set 1

Conventional gradient PCR was conducted using real-time PCR primers (table 2, set 5) with an annealing temperature range of 52℃ to 62℃. The optimum concentration of primers and probe were arrived at by checkerboard titration. The 25 µL reaction mixture comprised of 12.5 µL EmeraldAmp GT PCR Master mix (2X) (TaKaRa), 1 µL each of primers (10 pmol), 2 µL of DNA, and rest NFW to make up the volume. The cycling conditions were 95℃ for 2 min (initial denaturation), 34 cycles of 94℃ for 1 min (denaturation), gradient from 52℃ to 62℃ for 30 sec (annealing), and 72℃ for 1 min (polymerization) followed by single

cycle at 72°C for 5 min (final extension). Representative PCV2b, 2d and 2h samples were used. All the products were electrophoresed in two per cent agarose gels with ethidium bromide and visualized using a gel documentation system. The annealing temperature which gave the thickest band without primer dimer was selected for the real-time PCR.

The 20 µL TaqMan real-time PCR master mix contained 10 µL of 2X TaqMan^{R} Universal Master Mix II, with uracil-DNA glycosylase (UNG), (Applied Biosystems, USA), 4 µL, 1 µL and 2 µL of 10 pmol forward primer, reverse primer and TaqMan probe respectively, 2 µL of template DNA and rest NFW. The samples were run in a Step One plus real-time PCR (Applied Biosystems, Singapore) machine programmed to record the fluorescence of the reporter dye FAM. The cycling conditions are given in table 4.

Table 4. TaqMan real-time PCR cycling conditions

Sl. No.	Primer name	Cycling conditions	No. of cycles
1.	PCV2 PKD Taq FP/RP/TaqMan probe	50°C 2 min 95°C 10 min 95°C 15 sec, 57°C 30 sec	1 1 40

3.2.4.2.2 Standardization using real-time TaqMan PCR primer set 2

The newly synthesized forward primer and probe (table 2, set 6) were tested in conventional and real time PCR as described in 3.2.4.2.1 except that 0.5 µL (5 nmol) of primers and probes were used in real-time TaqMan PCR.

3.2.4.3 Determination of Sensitivity of TaqMan real-time PCR

3.2.4.3.1 Cloning and sequencing

3.2.4.3.1.1 Generation of blunt-ended products for cloning

Set up the blunting reaction on ice. The reaction mixture comprised of 10 µL 2X reaction buffer, 1 µL of PCR product, 1 µL of DNA blunting enzyme, and

NFW to make up the volume of 18 µL. The mixture was vortexed and centrifuged and incubated at 70°C for 5 minutes and chilled on ice.

3.2.4.3.1.2 Preparation of ligation mixture

The ligation mixture was prepared by using the CloneJET PCR cloning kit as per the manufacturer's guidelines. The ligation reaction was set up on the ice. The reaction mixture consisted of 10 µL of 2X reaction buffer, 1 µL of PCR Product, and 1 µL of pJETl.2/blunt cloning vector (50 ng/ µL), 1 µL of T4 DNA ligase (5 U/µL), and NFW to make up the volume to 20µL. The tube was vortexed and centrifuged for three to five sec. The ligation mixture was incubated at room temperature (22°C) for five min. the ligation mixture was used directly for transformation or kept at -20°C.

3.2.4.3.1.3 Preparation of *Escherichia coli* DH5α competent cells

Competent cells were prepared by the calcium chloride method according to the protocol of Sambrook and Russell (2001). The *E. coli* DH5α cells were streaked on an LB agar plate and incubated overnight at 37°C. A single colony of *E. coli* DH5α was picked up from the LB agar plate and inoculated into 10 mL LB broth and incubated overnight at 37°C in a shaker incubator. One millilitre of the overnight culture of *E. coli* DH5α cells was inoculated into 100 mL LB broth and incubated at 37°C in a shaker incubator till the OD_{600} of the culture reached 0.3-0.4. The bacterial culture was incubated at 4°C for 30 min and then transferred to a sterile pre-chilled 30 mL polypropylene centrifuge tube. The cells were pelleted by centrifugation at 6500 rpm for 10 min at 4°C. Discarded the supernatant and the cell pellet was resuspended in $1/10^{th}$ volume of ice-cold 0.1M $CaCl_2$ solution and incubated on ice for 30 min. The cells were harvested by centrifugation at 6000 rpm at 4°C for 10 min and resuspended in $1/25^{th}$ volume of ice-cold 0.1 M $CaCl_2$ and incubated at 4°C in ice for 10 min. Centrifugation was carried out at 5000 rpm at 4°C for 10 min. the supernatant was discarded and the cells were suspended in 850 µL of ice-cold 0.1 M $CaCl_2$ and 150 µL of 50 per cent glycerol in triple distilled water and mixed gently by pipetting. The cell suspension was aliquoted in 100 µL

volumes into pre-chilled sterile 1.5 mL microfuge tubes. The competent cells were stored at -80℃ until used.

3.2.4.3.1.4 Transformation of the competent cells

The transformation was carried out by the heat shock method according to the protocol of Sambrook and Russell (2001). One aliquot (100 µL) of the frozen competent cells was thawed on ice and 10 µL of the ligation mixture was added to it and the mixture was incubated on ice for 30 min. The cells were subjected to a heat shock at 42℃ for 90 sec and immediately transferred onto the ice for 10 min. Nine hundred microlitres of the LB broth without antibiotics was added to the tube and incubated at 37℃ for 3 h at 180 rpm in a shaker incubator. After taking from the shaker incubator, the tube was centrifuged at 6,000 rpm for 5 min. About 50 µL of the supernatant was retained. The pellet was disrupted by gentle pipetting and deposited onto an LB agar plate supplemented with 100 µg/mL ampicillin and spread using sterile spreaders. The plate was incubated at 37℃ for 16-20 h for the growth of the transformed bacteria. The colonies were screened for the recombinant plasmid DNA as described below.

3.2.4.3.1.5 Screening for identification of recombinants

Recircularised pJET 1.2/blunt vector contains a lethal gene that is disrupted by the ligation of a DNA insert into the cloning site. Hence cells with recombinant plasmids above grow, eliminating the need for expensive blue/white screening.

3.2.4.3.1.6 Confirmation of the recombinant plasmid DNA by PCR

About 2-3 colonies were picked up from the LB agar plate with sterile microtips and inoculated into 5 mL of LB broth containing 100 µg/mL ampicillin and incubated overnight at 37℃ in a shaker incubator at 150 rpm. After overnight incubation, 1 mL of the broth was centrifuged at 10,000 rpm for 5 min and the broth was pipetted out. The bacterial pellet was resuspended in 50 µL of NFW and kept in a boiling water bath for 5 min. Mixed the suspension by pipetting and centrifuged at 10,000 rpm for 5 min. One microliter of the supernatant was used as a template in the PCR for confirmation of the recombinant plasmid. The 20 µL PCR reaction

mix contained 10 µL of 2X EmeraldAmp GT PCR master mix, 0.4 µL each of 10 µM project 1.2 forward and reverse sequencing primers (supplied along with the kit), 1 µL of extracted plasmid, and the rest NFW to make up the volume. The PCR was carried out in a thermal cycler (Mastercycler Nexus gradient thermal cycler, Eppendorf, US) and the cycling conditions were initial denaturation at 95℃ for 3 min, followed by 25 cycles of 94℃ for 30 sec, 60℃ for 30 sec and 72℃ for 45 sec, and final extension at 72℃ for 1 min. The PCR products were subjected to submarine gel electrophoresis in one per cent agarose gel. The gel was examined in a gel documentation system under UV to confirm the presence of the insert and the size of the product as described in 3.2.2.1.3.

3.2.4.3.1.7 Isolation of plasmid DNA

From the overnight broth, the culture was found to be positive for the recombinant *E. coli*, and the plasmid DNA was isolated using the alkaline lysis method (Sambrook and Russell, 2001). About 1.5 mL of the overnight culture was pelleted by centrifugation at maximum speed for 30 sec at 4℃. Discarded the supernatant and resuspended the bacterial pellet in 100 µL of ice-cold alkaline lysis solution 1, by vigorous vortexing. Added 200 µL of freshly prepared alkaline lysis solution 2 to each bacterial suspension and mixed the contents by inverting rapidly 5 times. Stored the tube on ice. Added 150 µL of alkaline lysis solution 3 and dispersed it through bacterial suspension. Stored tube on ice for 2-5 min. Centrifuged the bacterial lysate at maximum speed for 5 min at 4℃ in a microfuge and transferred the supernatant to a fresh tube. Precipitated nucleic acids from the supernatant by adding two volumes of ethanol at room temperature. Mixed the solution by vortexing and allowed it to stand for 2 min at room temperature. Collected precipitated nucleic acid by centrifugation at maximum speed for 5 min at 4℃ and removed the supernatant by gentle aspiration. Made the tube stand in an inverted position on a paper towel, to allow all of the fluid to drain away and remove any drop of fluid adhering to the walls of the tube. Added 1 mL of 70 per cent ethanol to the pellet and inverted the closed tube several times. Recovered DNA by centrifugation at maximum speed for 2 min at 4℃. Removed all supernatant by

aspiration and also removed any beads of ethanol has evaporated and no fluid is visible in the tube. Dissolved nucleic acids in 30 µL of NFW and vortexed for a few seconds. Stored DNA solution at -20℃ after checking the concentration of the plasmid DNA in ng/µL by Qubit 4 fluorometer as described in 3.2.4.3.1.8.

Glycerol stock was prepared with 700 µL of the positive LB broth culture and 300 µL of 50 per cent glycerol solution. This solution was mixed gently by inversion and kept frozen at -20℃ for future use.

3.2.4.3.1.8 Estimation of concentration of recombinant plasmid by Qubit 4 fluorometer

Two assay tubes for the standards and one assay tube for the sample was set up. Qubit™ working solution was prepared by diluting the Qubit™ reagent 1:200 in Qubit™ buffer. Prepared 200 µL of working solution for each standard and sample. Standard assay tube was prepared by adding 190 µL of working solution and 10 µL of standard from the kit. The sample assay tube was prepared by adding 180 µL of working solution and 20 µL of the sample. Vortexed all the tubes for 2–3 seconds and incubated the tubes for 2 minutes at room temperature. Inserted the tubes in the Qubit™ Fluorometer and readings were taken.

3.2.4.3.1.9 Sequencing of recombinant plasmid

The plasmids from the recombinant *E. coli* clones carrying the ORF2 fragment was sequenced by using T7 promoter sequencing primer at M/s AgriGenome Labs Private Limited, Kakkanad, Cochin, Kerala.

3.2.4.3.2 Calculation of copy number

The plasmid DNA concentration was used to calculate the copy number of plasmid DNA using the following formula:

No. of copies = (amount x 6.022 x 10^{23})/length of plasmid with insert x 1 x 10^{9} x 650)

This calculation was based on the assumption that the average weight of a base pair (bp) is 650 Daltons *i.e.,* one mole of a bp weighs 650 g, and that the molecular weight of any double-stranded DNA template can be estimated by taking the product of its length (in bp) and 650. The inverse of the molecular weight is the number of moles of template present in one gram of material. Using Avogadro's number, 6.022×10^{23} molecules/mole, the number of molecules of the template per gram can be calculated:

Mol/g (x) molecules/mol = molecules/g.

Finally, the number of molecules or number of copies of the template in the sample was estimated by multiplying by $1x10^{9}$ to convert to ng and then multiplying by the amount of template (in ng).

3.2.4.3.3 Sensitivity of TaqMan real-time PCR based on copy number

Serial 10-fold dilutions (10^{-1} to 10^{-10}) of the purified plasmid were prepared and it was used to determine the sensitivity of TaqMan Real-Time PCR based on copy number. Serial dilutions were made in NFW. The reactions were put in duplicate as described in step 3.2.4.2 with 2 µL of 10^{-1} to 10^{-10} dilution of plasmid DNA as a template. The cycling conditions were described in table 4. The highest dilution of plasmid DNA which could be detected in the real-time PCR was determined from the C_t values obtained.

3.2.4.3.4 Statistical analysis

The sensitivity, specificity, and accuracy of the TaqMan Real-Time PCR were compared with the ORF2-based PCR and were calculated using the following formula:

Sensitivity: $a / (a+c)$

Specificity: $d / (b+d)$

Accuracy: $a+d / (a+b+c+d)$

a is the number of samples positive by both i.e., test to be compared and gold standard test, b is the number of samples positive by standard test whereas negative by test to be compared, c is the number of samples negative by standard test and positive by test to be compared and d is the number of samples negative by both.

The results obtained from the test were analysed for the percentage of agreement with ORF2-based PCR with the use of Kappa statistics using SPSS version 24.0 software. Kappa statistics are a decimal measure of agreement between two tests, especially in the absence of a standard, and are defined as kappa or κ.

K value <0.1 indicates poor agreement

K value between 0.1 and 0.2 indicates slight agreement

K value between 0.21 and 0.4 indicates fair agreement

K values 0.41 and 0.6 indicate moderate agreement

K value 0.61 and 0.8 indicates substantial agreement

K value >0.81 indicates perfect agreement

Results

4. RESULTS

4.1 SAMPLE COLLECTION

A total of 62 samples were collected during necropsy of pigs that have died exhibiting any of the symptoms of PCV-2 infection such as respiratory illness, gradual wasting, thriftiness, rough hair coat, polypnoea, dyspnoea, pallor, diarrhoea, icterus *etc*. All tissue samples obtained were of domestic pigs. The details of the tissue samples collected are given in Table 1.

4.2 MOLECULAR CONFIRMATION OF PCV2 IN THE TISSUE SAMPLES

4.2.1 Detection of PCV2 by conventional PCR

Total DNA was extracted from tissue samples using the GeneJET DNA purification kit (step 3.2.2.1.1). The obtained DNA was subjected to conventional PCR targeting 481 bp fragment of the ORF2 of PCV2.

Of the 62 samples tested by conventional PCR based on ORF2, 36 (58.0 per cent) were positive and 26 (41.94 per cent) were negative for PCV2 (Table 5 and 6). Amplicon with a product size of 481 bp was observed on agarose gel electrophoresis (plate 2).

Table 5. Result of the samples tested for PCV2 by conventional PCR targeting ORF2.

SI. No.	Sample No.	Result
1	16/MIB/2021	-
2	17/MIB/2021	+
3	33/MIB/2021	+
4	34/MIB/2021	+
5	47/MIB/2021	+
6	78/MIB/2021	+
7	118/MIB/2021	+
8	122/MIB/2021	+
9	123/MIB/2021	+
10	124/MIB/2021	+
11	127/MIB/2021	+

12	128/MIB/2021	+
13	129/MIB/2021	+
14	132/MIB/2021	-
15	133/MIB/2021	-
16	146/MIB/2021	+
17	168/MIB/2021	+
18	181/MIB/2021	-
19	248/MIB/2021	+
20	256/MIB/2021	+
21	260/MIB/2021	-
22	261/MIB/2021	+
23	269/MIB/2021	+
24	272/MIB/2021	-
25	273/MIB/2021	-
26	312/MIB/2021	+
27	316/MIB/2021	+
28	353/MIB/2021	+
29	370/MIB/2021	+
30	371/MIB/2021	+
31	384/MIB/2021	-
32	394/MIB/2021	-
33	399/MIB/2021	-
34	427/MIB/2021	-
35	431/MIB/2021	-
36	432/MIB/2021	+
37	437/MIB/2021	-
38	454/MIB/2021	+
39	35/MIB/2022	-
40	40/MIB/2022	-
41	42/MIB/2022	+
42	45/MIB/2022	+
43	58/MIB/2022	-
44	62/MIB/2022	-
45	71/MIB/2022	+
46	72/MIB/2022	+
47	73/MIB/2022	+
48	88/MIB/2022	-
49	103/MIB/2022	+
50	121/MIB/2022	+
51	169/MIB/2022	+
52	188/MIB/2022	+
53	189/MIB/2022	-
54	192/MIB/2022	+
55	194/MIB/2022	-

56	242/MIB/2022	-
57	257/MIB/2022	-
58	89/MIB/2022	-
59	148/MIB/2022	-
60	186/MIB/2022	-
61	248/MIB/2022	+
62	294/MIB/2022	-

Table 6. Result of testing samples for PCV2 by conventional PCR based on ORF2.

Number of samples tested	Number of samples positive	Number of samples negative
62	36 (58.06%)	26 (41.94%)

4.3 GENOTYPING OF PCV2

Representative samples which were positive in the conventional PCR for ORF2 were selected for genotyping.

4.3.1 Confirmation of PCV2 by sequencing and assembly of whole genome

The whole genome of PCV2 in the selected positive samples were amplified using three sets of primers that could amplify overlapping regions of the genome as described by Anoopraj *et al.* (2015). Amplicons of 661 bp, 765 bp, and 676 bp were obtained. The PCR amplicons were subjected to gel purification and sequencing. The sequences were subjected to BLAST analysis for confirmation of the sequences obtained. The complete genomes were assembled from the three fragments using Emboss Merger program. Accession numbers were obtained by uploading the sequences in GeneBank using the Bankit submission tool (Table 7).

Plate 2. Agarose gel showing the 481 bp amplicons of ORF2 of PCV2

Lane 1: 100 bp ladder

Lane 2 to 5: Clinical samples

Lane 6: No template control

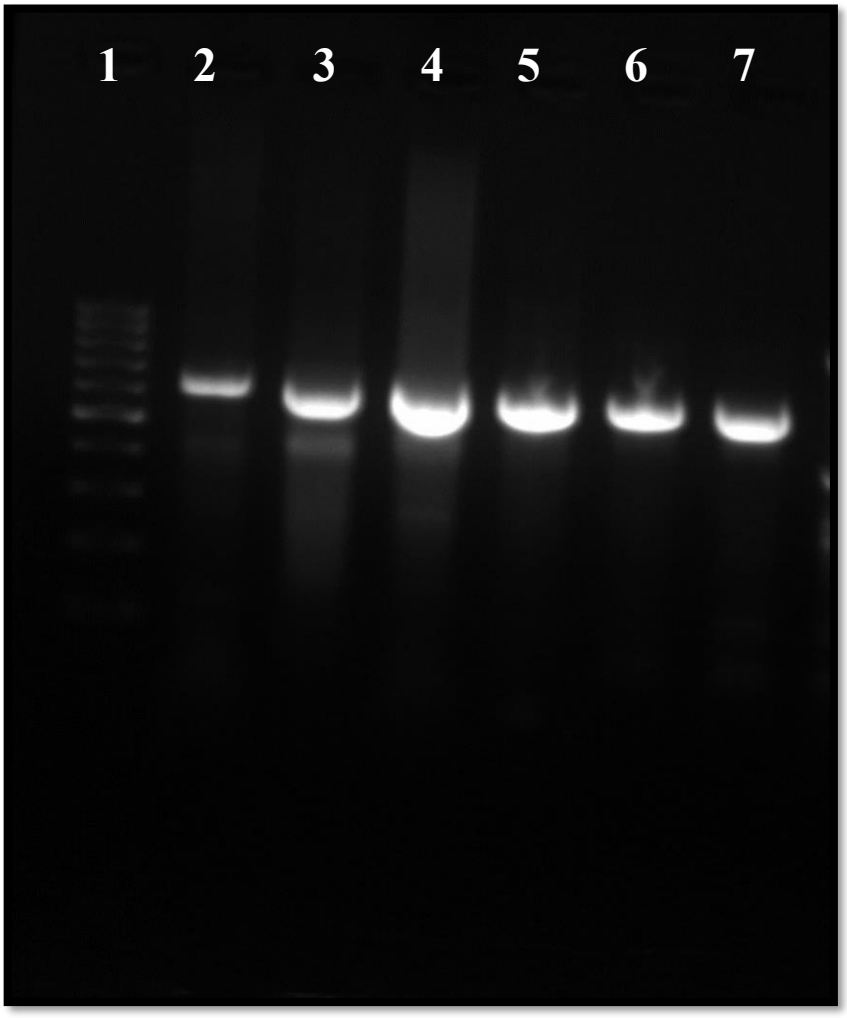


Plate 3. Agarose gel showing the 661 bp amplicons of PCV2

Ladder 1: 100 bp ladder

Ladder 2 to 7: Clinical samples (661 bp PCR amplicons)

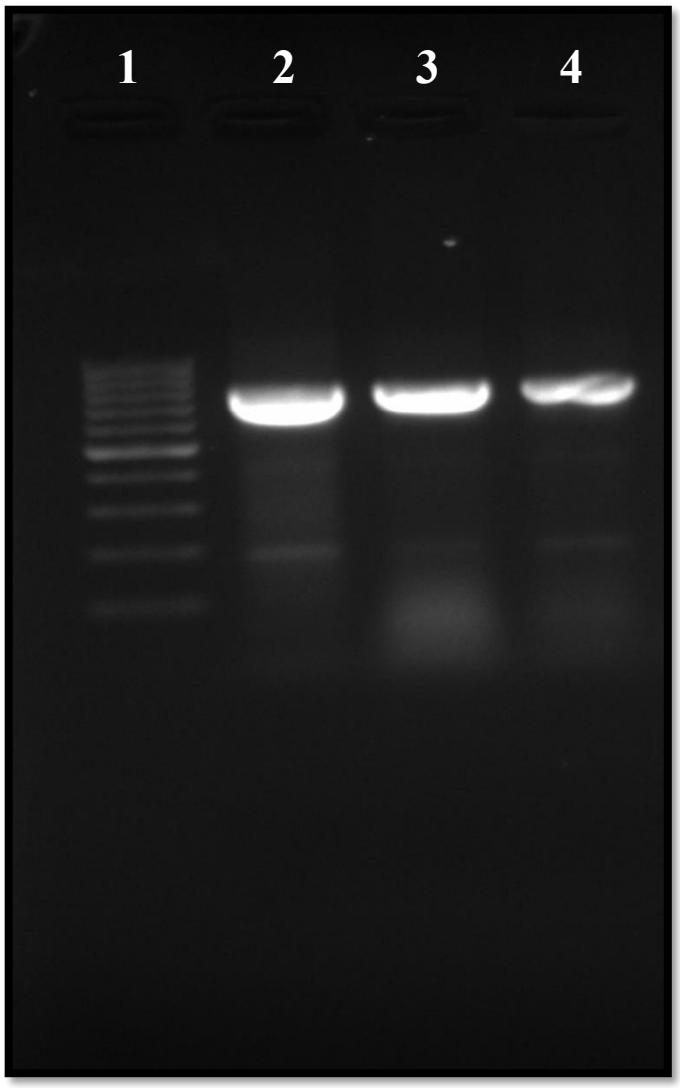


Plate 4. Agarose gel showing the 765 bp amplicons of PCV2

Ladder 1: 100 bp ladder

Ladder 2 to 4: Clinical samples (765 bp PCR amplicons)

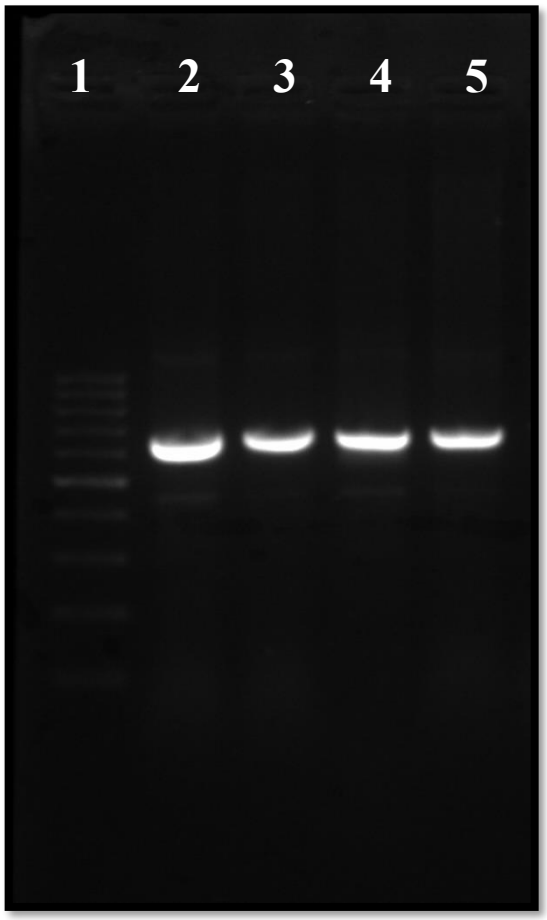


Plate 5. Agarose gel showing the 676 bp amplicons of PCV2

Lane 1: 100 bp ladder

Lane 2 to 5: Clinical samples (676 bp PCR amplicons)

Table 7. Accession numbers obtained for the sequences

Sl. No.	Sample No.	Accession No.
1	33/MIB/2021	OP455989
2	47/MIB/2021	OP455990
3	78/MIB/2021	OP455991
4	118/MIB/2021	OP455992
5	122/MIB/2021	OP455993
6	127/MIB/2021	OP455994
7	128/MIB/2021	OP455995
8	146/MIB/2021	OP455996
9	248/MIB/2021	OP455997
10	256/MIB/2021	OP455998
11	261/MIB/2021	OP455999

The results of the BLAST analysis of the whole genomes revealed that the isolates were similar to PCV2 isolates from India and abroad. On BLAST analyses all the sequences showed a high degree of homology with the other isolates from Kerala (99.14-99.92 per cent identity) except isolates 78/MIB/2021, 127/MIB/2021, and 128/MIB/2021 which had more identity with Uttar Pradesh isolates (99.00-99.26 per cent identity). Foreign isolates which showed a high percentage identity were of China (99.77-99.89 per cent identity) except isolates 33/MIB/2021, 127/MIB/2021, and 128/MIB/2021 which had more identity with Vietnam isolates (99.65-99.72 per cent identity).

Table 8. Nucleotide identity based on PCV2 sequences

Sl. No.	Sample No.	Closest match with the Indian sequence	State	% Identity	Closest match with a foreign sequence	Country	% Identity
1	33/MIB/ 2021	MW268738	Kerala	99.14	JQ181600	Vietnam	99.72
2	47/MIB/ 2021	MW268739	Kerala	99.61	MF589542	China	99.77
3	78/MIB/ 2021	MK035433	Uttar Pradesh	99.26	KF742549	China	99.83
4	118/MIB /2021	MW268738	Kerala	99.77	ON012565	China	99.83

5	122/MIB /2021	MT828905	Kerala	99.92	MN170523	China	99.77
6	127/MIB /2021	MK035432	Uttar Pradesh	99.00	KM042400	Vietnam	99.65
7	128/MIB /2021	MK035432	Uttar Pradesh	99.04	KM042400	Vietnam	99.66
8	146/MIB /2021	MW268739	Kerala	99.15	MH341493	China	99.89
9	248/MIB /2021	MW268738	Kerala	99.76	MH341493	China	99.89
10	256/MIB /2021	MW268738	Kerala	99.84	ON012565	China	99.89
11	261/MIB /2021	MW268738	Kerala	99.83	KX960933	China	99.83

4.3.2 Phylogenetic analysis of PCV2 sequences

The phylogenetic and molecular evolutionary analyses of the PCV2 sequences were carried out with the help of the MEGA X software. For this, representative PCV2 sequences from India and various other countries were downloaded from GeneBank.

Three phylogenetic trees based on the partial ORF2, complete ORF2 and complete genome sequences of the 11 PCV2 strains sequenced were constructed together with the reference sequences deposited in the GeneBank database presenting PCV genotypes from India, China, Vietnam, Malaysia, and South Korea. All the phylogenetic trees grouped the eight samples *viz*., 33, 47, 118, 122, 146, 248, 256, and 261/MIB/2021 into genotype 2d and cluster 13. Sample 78/MIB/2021 was grouped into genotype 2b and cluster 11, and samples 127/MIB/2021 and 128/MIB/2021 were grouped into genotype 2h and cluster 18. Hence, among all 11 isolates, 1 comes under PCV2b (9.09 per cent), 8 belong to PCV2d (72.72 per cent), and 2 exist in PCV2h (18.18 per cent) indicating the predominance of PCV2d strain.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Porcine circovirus 2 isolate 75/MIB/2020, complete genome	Porcine circovirus 2	2351	3252	100%	0.0	99.92%	1766	MT828905.1
Porcine circovirus 2 isolate 169/MIB/2020, complete genome	Porcine circovirus 2	2348	3232	100%	0.0	99.84%	1767	MW268738.1
Porcine circovirus 2 isolate 181/MIB/2020, complete genome	Porcine circovirus 2	2337	3220	100%	0.0	99.69%	1767	MW268739.1

Fig 1. BLAST analysis of whole genome sequences under the study (India)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Porcine circovirus 2 isolate Han8, complete genome	Porcine circovirus 2	3236	3236	100%	0.0	99.72%	1767	JQ181600.1
Porcine circovirus 2 strain PCV2/CH/GX/469/2015, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	MN052978.1
Porcine circovirus 2 isolate SD-4, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	MH920545.1
Porcine circovirus 2 strain KSU-IA-2018-PCV2-45, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	MK504418.1
Porcine circovirus 2 strain KSU-KS-2016-PCV2-15, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	MK504395.1
Porcine circovirus 2 strain KSU-KS-2016-PCV2-11, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	MK504391.1
Porcine circovirus 2 strain KSU-MN-2017-PCV2-8, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	MK504388.1
Porcine circovirus 2 isolate XA07174, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	MK347358.1
Porcine circovirus 2 strain GXYL1401, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	MH465476.1
Porcine circovirus 2 strain GXNN141225, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	MH756616.1
Porcine circovirus 2 strain SCADC17-YA2, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	MH094770.1
Porcine circovirus 2 strain NJ1, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	KX247809.1
Porcine circovirus 2 isolate Hau7, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	KT336599.1
Porcine circovirus 2 strain SXPY, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	KX352160.1
Porcine circovirus 2 isolate YiY-3-36-1, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	KU317493.1
Porcine circovirus 2 isolate YiY-3-28-1, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	KU317483.1
Porcine circovirus 2 isolate LY2119, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	OL504750.1
Porcine circovirus 2 isolate LC001 ORF1 and ORF2 genes, complete cds	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	MZ151445.1
Porcine circovirus 2 strain PCV2-CH-SDBZL-2021, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	OK170034.1
Porcine circovirus 2 strain Y-2, complete genome	Porcine circovirus 2	3230	3230	100%	0.0	99.66%	1767	KF027492.1

Fig 2. BLAST analysis of whole genome sequences under the study (Foreign)

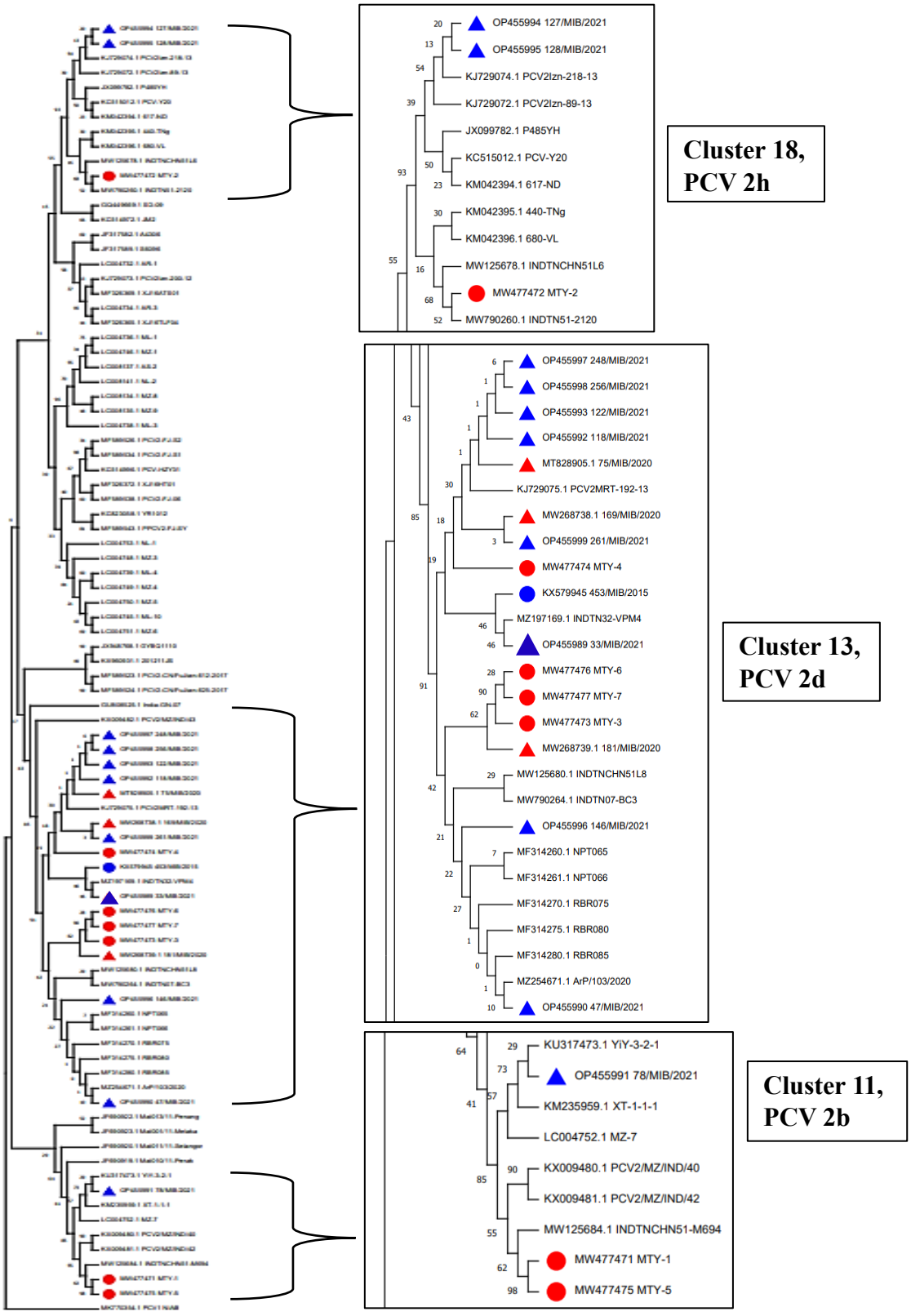


Fig 3. Phylogenetic tree created using partial ORF2 (1107-1557 nt) sequences of PCV2 from Kerala, India, and few Asian countries. Legend: the symbols "filled triangle" and "filled circle" depicts the isolates from North Kerala and Central Kerala respectively. Boot strap replicates = 1000. Evolutionary distances between sequences were calculated by the Kimura 2 parameter method

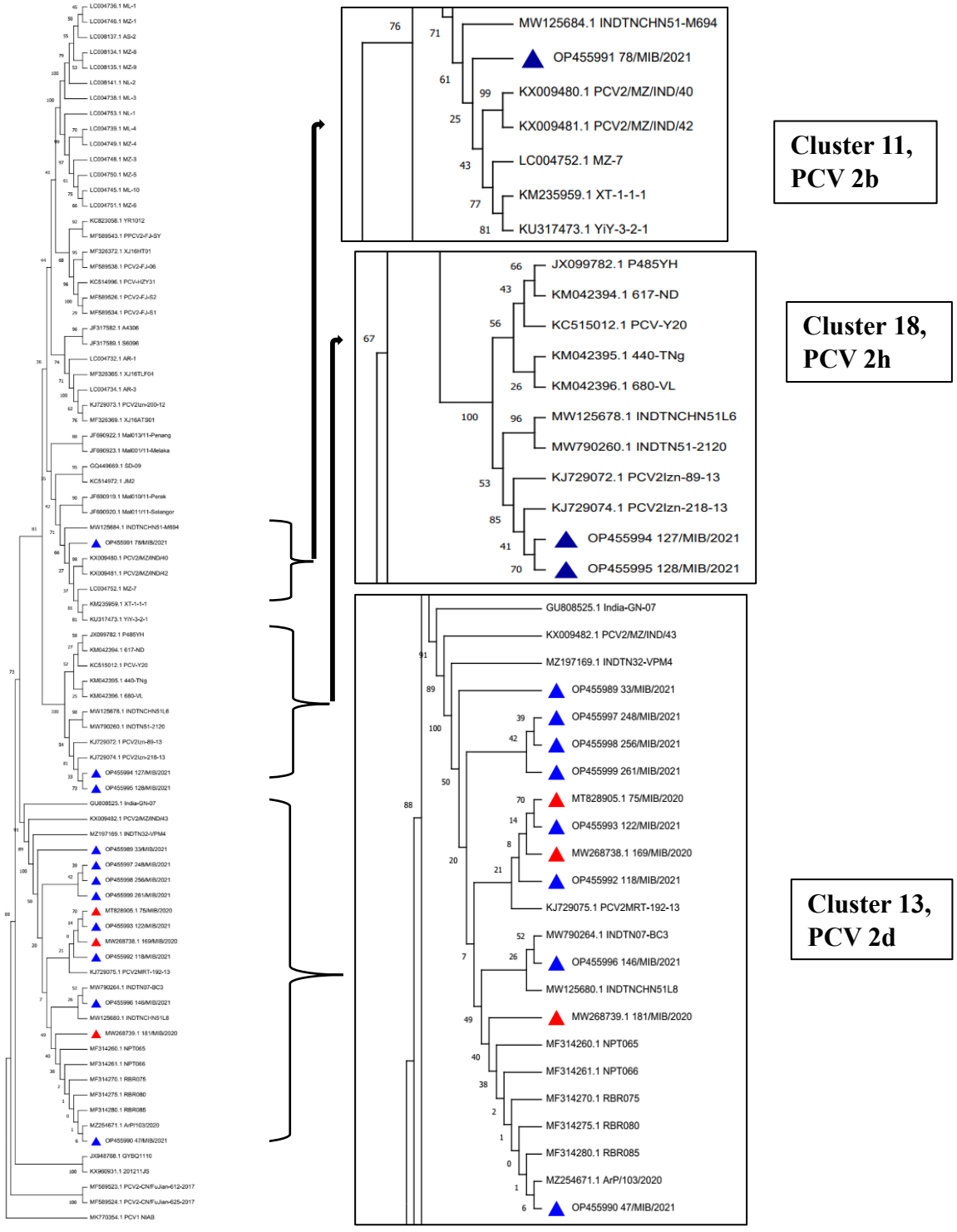


Fig 4. Phylogenetic tree constructed from nucleotide sequences of complete ORF2 of PCV2 from Kerala and abroad. Legend: The symbols 'filled blue triangle' and 'filled red triangle' depicts isolates under study and other isolates from Kerala respectively. Boot strap replicates = 1000. Evolutionary distances between sequences were calculated by the Kimura 2 parameter method.

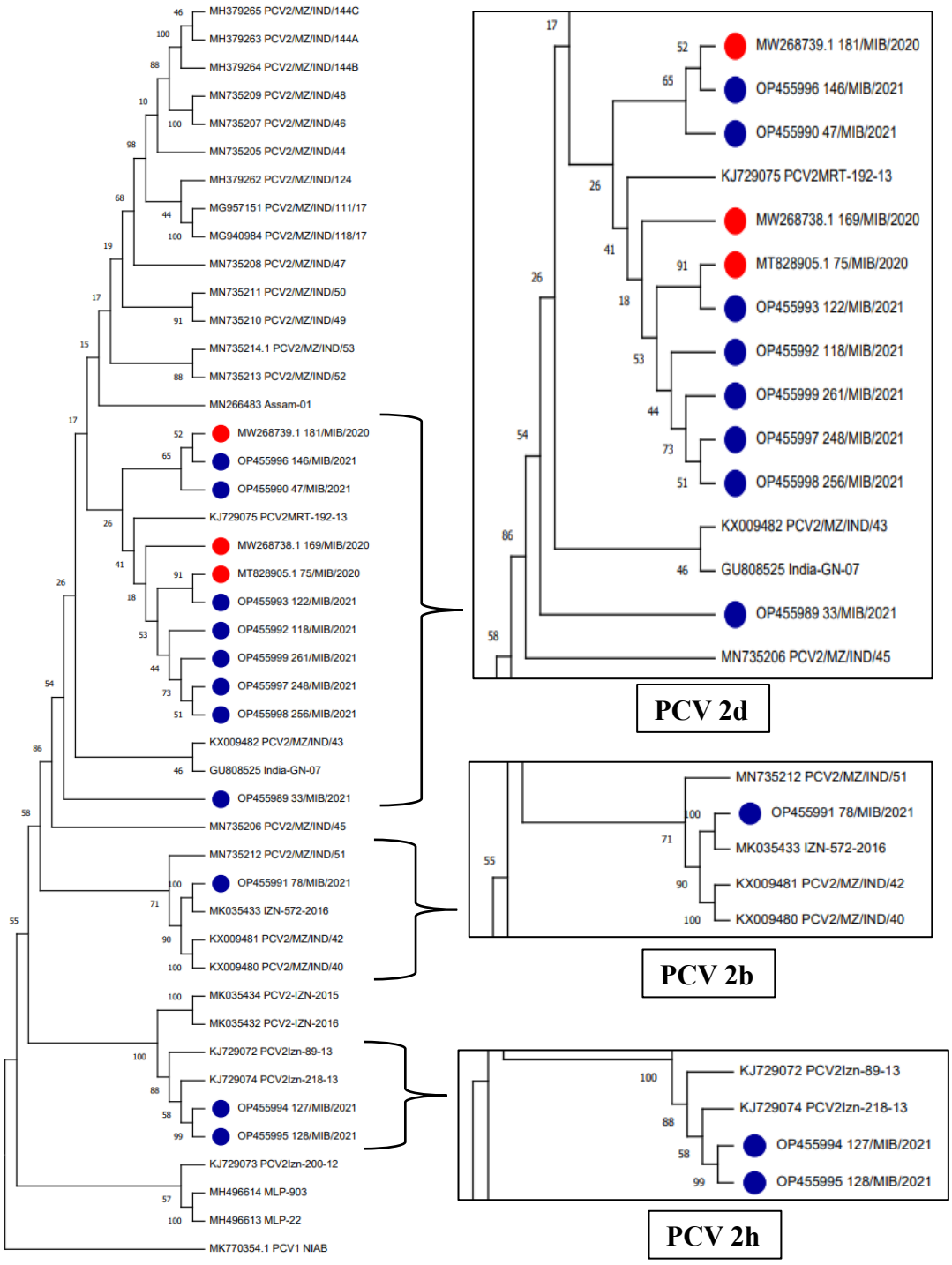


Fig 5. Phylogenetic tree constructed from nucleotide sequences complete genome of PCV2 from Kerala and India. Legend: The symbols 'filled blue circle' and 'filled red circle' depicts isolates under study and other isolates from Kerala respectively. Boot strap replicates = 1000. Evolutionary distances between sequences were calculated by the Tamura-Nei parameter method.

4.4 AMINO ACID ANALYSIS OF ORF2 OF PCV2

On analysis of the amino acid sequences of ORF2, it was observed that except for isolates 78 and 128/MIB/2021, the other Kerala isolates were conserved. At positions 8, 57, 89 and 90, 78/MIB/2021 had Tyrosine (Y), Isoleucine (I), Arginine (R) and Serine (S) whereas all the other Kerala isolates Phenylalanine (F), Valine (V), Leucine (L) and Threonine (T). As far as isolate 128/MIB/2021 is concerned, it had Proline (P), Alanine (A) and Histidine (H) at positions 131, 191 and 232, and all the other Kerala isolates had Threonine (T), Glycine (G) and Asparagine (N). At positions 53, 68 and 134, isolates 78 and 128/MIB/2021 had Phenylalanine (F), Alanine (A) and Threonine (T) and all other Kerala isolates had Isoleucine (I) at position 53 and, Asparagine (N) at the other two positions. The most variable position among Kerala isolates was 169, where 181/MIB/2020, 47/MIB/2021 and 146/MIB/2021 had Glycine (G) which is similar to Tamil Nadu isolates, whereas, 33/MIB/2021, 78/MIB/2021, and 128/MIB/2021 had Serine (S) which is similar to that recorded in isolates from Tamil Nadu, Uttar Pradesh and North Eastern states of India. The third type of amino acid noticed in this position in Kerala isolates was Arginine (R) that has been recorded in isolates from Uttar Pradesh and Mizoram. At position 234, all the Kerala sequences had Lysine (K) except 118/MIB/2021 which had Asparagine (N), and 146/MIB/2021 which had Glutamine (Q). The presence of Asparagine (N) in position 234 has not been recorded in any of the other sequences from Kerala and India. The nucleotide and amino acid changes of ORF2 sequences are depicted in Tables 9 and 10 respectively.

Table 9. Variations in nucleotides in capsid protein encoded by ORF2 of PCV2 from Kerala and India

Nt. Pn.	**75/ MIB /20**	**33/ MIB /21**	**47/ MIB /21**	**78/ MIB /21**	**118/ MIB /21**	**122/ MIB /21**	**128/ MIB /21**	**146/ MIB /21**	**248/ MIB /21**	**256/ MIB /21**	**261/ MIB /21**	**Change in NT**	**Mutation**
23	T	-	-	A	-	-	-	-	-	-	-	T-A	Transversion
27	C	-	-	G	-	-	-	-	-	-	-	C-G	Transversion
31	C	-	-	A	-	-	-	-	-	-	-	C-A	Transversion
60	A	C	C	C	C	-	C	C	C	C	C	C-A	Transversion
81	C	-	-	-	-	-	-	-	A	A	A	C-A	Transversion
129	C	-	-	-	-	-	-	A	-	-	-	C-A	Transversion
157	A	-	-	T	-	-	T	-	-	-	-	A-T	Transversion
162	T	-	-	A	-	-	A	-	-	-	-	T-A	Transversion
169	G	-	-	A	-	-	-	-	-	-	-	G-A	Transition
171	C	-	-	-	-	-	G	-	-	-	-	C-G	Transversion
202	A	-	-	G	-	-	G	-	-	-	-	A-G	Transition
203	A	-	-	C	-	-	C	-	-	-	-	A-C	Transversion
204	T	-	-	G	-	-	G	-	-	-	-	T-G	Transversion
222	T	-	-	C	-	-	-	-	-	-	-	T-C	Transition
231	T	-	-	-	-	-	C	-	-	-	-	T-C	Transition
234	T	-	-	-	C	-	-	-	-	-	-	T-C	Transition
237	T	-	-	-	-	-	C	-	-	-	-	T-C	Transition
252	G	-	-	-	-	-	-	A	-	-	-	G-A	Transversion
266	T	-	-	G	-	-	-	-	-	-	-	T-G	Transversion
268	A	-	-	T	-	-	-	-	-	-	-	T-A	Transversion
297	G	-	-	A	-	-	A	-	-	-	-	G-A	Transition
330	A	-	-	G	-	-	-	-	-	-	-	A-G	Transition
354	G	-	-	-	-	-	T	-	-	-	-	G-T	Transversion

357	C	-	-	-	-	-	A	-	-	-	-	C-A	Transversion
362	C	-	-	G	-	-	-	-	-	-	-	C-G	Transversion
391	A	-	-	-	-	-	C	-	-	-	-	A-C	Transversion
401	A	-	-	C	-	-	C	-	-	-	-	A-C	Transversion
402	T	-	-	A	-	-	A	-	-	-	-	T-A	Transversion
408	A	-	-	C	-	-	G	-	-	-	-	A-C/G	Transversion
411	C	-	-	-	-	-	T	-	-	-	-	C-T	Transition
417	C	-	-	-	-	-	T	-	-	-	-	C-T	Transition
477	G	-	-	-	-	-	G	-	-	-	-	G-C	Transversion
489	G	-	-	-	-	-	C	-	-	-	-	C-G	Transversion
498	C	-	-	-	-	-	T	-	-	-	-	C-T	Transition
501	T	-	-	A	-	-	-	-	-	-	-	T-A	Transversion
505	A	-	G	T	-	-	T	G	-	-	-	A-T/G	Transversion/Transition
506	G	-	-	C	-	-	-	-	-	-	-	G-C	Transversion
507	G	C	-	C	-	-	C	-	-	-	-	G-C	Transversion
510	A	-	-	T	-	-	-	-	-	-	-	A-T	Transversion
513	C	-	-	T	-	-	T	-	-	-	-	C-T	Transition
528	C	-	-	A	-	-	G	-	-	-	-	C-A/G	Transversion/Transition
531	T	-	-	C	-	-	T	-	-	-	-	T-C	Transition
540	A	-	-	A	-	-	G	-	-	-	-	A-G	Transition
546	A	-	-	G	-	-	G	-	-	-	-	A-G	Transition
549	C	-	-	G	-	-	T	-	-	-	-	C-G/T	Transversion/Transition
558	A	-	-	-	-	-	G	-	-	-	-	A-G	Transition
567	T	-	-	-	-	-	C	-	-	-	-	T-C	Transition
568	A	-	-	-	-	-	T	-	-	-	-	A-T	Transversion

572	G	-	-	-	-	-	C	-	-	-	-	G-C	Transversion
579	A	-	-	-	-	-	G	-	-	-	-	A-G	Transition
585	T	-	-	C	-	-	C	-	-	-	-	T-C	Transition
630	C	-	-	A	-	-	-	-	-	-	-	C-A	Transversion
643	A	-	-	G	-	-	G	-	-	-	-	A-G	Transition
694	A	-	-	-	-	-	C	-	-	-	-	A-C	Transversion
702	G	-	-	-	C	-	-	-	-	-	-	G-C	Transversion

Table 10. Variations in amino acids in capsid protein encoded by ORF2 of PCV2 from Kerala and India

*****Stop Codon

Sl. No.	Sample No./Isolate name	State	Accession No.	Amino acid positions in ORF2 (Consensus amino acid in Kerala isolates)											
				8 (F)	53 (I)	57 (V)	68 (N)	89 (L)	90 (T)	131 (T)	134 (N)	169 (R)	191 (G)	232 (N)	234 (K)
1	75/MIB/2020	Kerala	MT828905	-	-	-	-	-	-	-	-	-	-	-	-
2	181/MIB/2020	Kerala	MW268739	-	-	-	-	-	-	-	-	G	-	-	-
3	169/MIB/2020	Kerala	MW268738	-	-	-	-	-	-	-	-	-	-	-	-
4	33/MIB/2021	Kerala	OP455989	-	-	-	-	-	-	-	-	S	-	-	-
5	47/MIB/2021	Kerala	OP455990	-	-	-	-	-	-	-	-	G	-	-	-
6	78/MIB/2021	Kerala	OP455991	Y	F	I	A	R	S	-	T	S	-	-	-
7	118/MIB/2021	Kerala	OP455992	-	-	-	-	-	-	-	-	-	-	-	N
8	122/MIB/2021	Kerala	OP455993	-	-	-	-	-	-	-	-	-	-	-	-
9	128/MIB/2021	Kerala	OP455995	-	F	-	A	-	-	P	T	S	A	H	-
10	146/MIB/2021	Kerala	OP455996	-	-	-	-	-	-	-	-	G	-	-	Q
11	248/MIB/2021	Kerala	OP455997	-	-	-	-	-	-	-	-	-	-	-	-
12	256/MIB/2021	Kerala	OP455998	-	-	-	-	-	-	-	-	-	-	-	-
13	261/MIB/2021	Kerala	OP455999	-	-	-	-	-	-	-	-	-	-	-	-
14	PCV2/MZ/IND/40	Mizoram	KX009480	Y	F	I	A	R	S	-	T	S	-	-	*
15	PCV2/MZ/IND/42	Mizoram	KX009481	Y	F	I	A	R	S	-	T	S	-	-	*
16	MZ-7	Mizoram	LC004752	Y	F	I	A	R	S	-	T	S	-	-	*
17	India-GN-07	Uttar Pradesh	GU808525	Y	F	-	A	-	-	-	-	-	-	-	-
18	PCV2MRT-192-13	Uttar Pradesh	KJ729075	-	-	-	-	-	-	-	-	-	-	-	-
19	PCV2/MZ/IND/43	Mizoram	KX009482	Y	-	-	-	-	-	-	-	-	-	-	-
20	PCV2IZN-218-13	Uttar Pradesh	KJ729074	-	F	-	A	-	-	P	T	S	A	H	-

21	PCV2IZN-89-13	Uttar Pradesh	KJ729072	-	F	-	A	-	-	P	T	S	A	H	-
22	PCV2IZN-200-12	Uttar Pradesh	KJ729073	-	F	-	A	I	S	I	T	S	R	K	*
23	AR-1	Northeast India	LC004732	Y	F	-	A	I	S	P	T	S	R	K	*
24	AR-3	Northeast India	LC004734	Y	F	-	A	I	S	I	T	S	R	K	*
25	ML-1	Northeast India	LC004736	Y	F	-	A	-	-	I	T	S	-	-	-
26	ML-3	Northeast India	LC004738	Y	F	-	A	-	-	I	T	S	-	-	E
27	ML-4	Northeast India	LC004739	Y	F	-	A	-	-	I	T	S	-	-	-
28	ML-10	Northeast India	LC004745	Y	F	-	A	-	-	I	T	S	-	-	-
29	MZ-1	Northeast India	LC004746	Y	F	-	A	-	-	I	T	S	-	-	-
30	MZ-3	Northeast India	LC004748	Y	F	-	A	-	-	I	T	S	-	-	-
31	INDTNCHN51-M694	Tamil Nadu	MW125684	Y	F	I	A	P	S	-	T	S	-	-	-
32	INDTN32-VPM4	Tamil Nadu	MZ197159	Y	-	-	-	-	-	-	-	G	-	-	-
33	INDTNCHN51L8	Tamil Nadu	MW125680	-	-	-	-	-	-	-	-	G	-	-	-
34	INDTN07-BC3	Tamil Nadu	MW790264	-	-	-	-	-	-	-	-	G	-	-	Q
35	INDTN51-2120	Tamil Nadu	MW790260	-	F	-	A	-	-	P	T	S	A	H	-
36	ArP/103/2020	Tamil Nadu	MZ254671	-	-	-	-	-	-	-	-	G	-	-	-
37	INDTNCHN51L6	Tamil Nadu	MW125678	-	F	-	A	-	-	P	T	S	A	H	-
Other Indian isolates				F, Y	I, F	I, V	N, A	L, R, I, P	S, T	T, P, I	T, N	S, R, G	G, A R	N, H K	K, E Q

4.5 TAQMAN REAL-TIME PCR FOR DETECTION OF PCV2

4.5.1 Standardization of TaqMan real-time PCR

The standardization of TaqMan real-time PCR was carried out as described in 3.2.4.2. In conventional PCR, the real-time PCR primers (set 5) amplified the expected 81 bp fragment of ORF2 of PCV2d, 2b and 2h (Plate 6). However, the probe was not able to detect any of these genotypes. The modified forward primer and TaqMan probe that was designed to detect 2d and the reverse primer of Set 6 were able to detect PCV2 isolates (Plate 7).

4.5.2. Testing of samples by TaqMan real-time PCR

TaqMan real-time PCR was carried out to detect the presence of PCV2, using the DNA of 40 (20 positive and 20 negative) samples and their respective Ct values are noted. The results of TaqMan real-time PCR in comparison to the results of gel-based PCR are given in Table 11 and 12. The percentage positivity of TaqMan real-time PCR was estimated to be 57.5 and that of ORF2-based PCR was estimated to be 50.

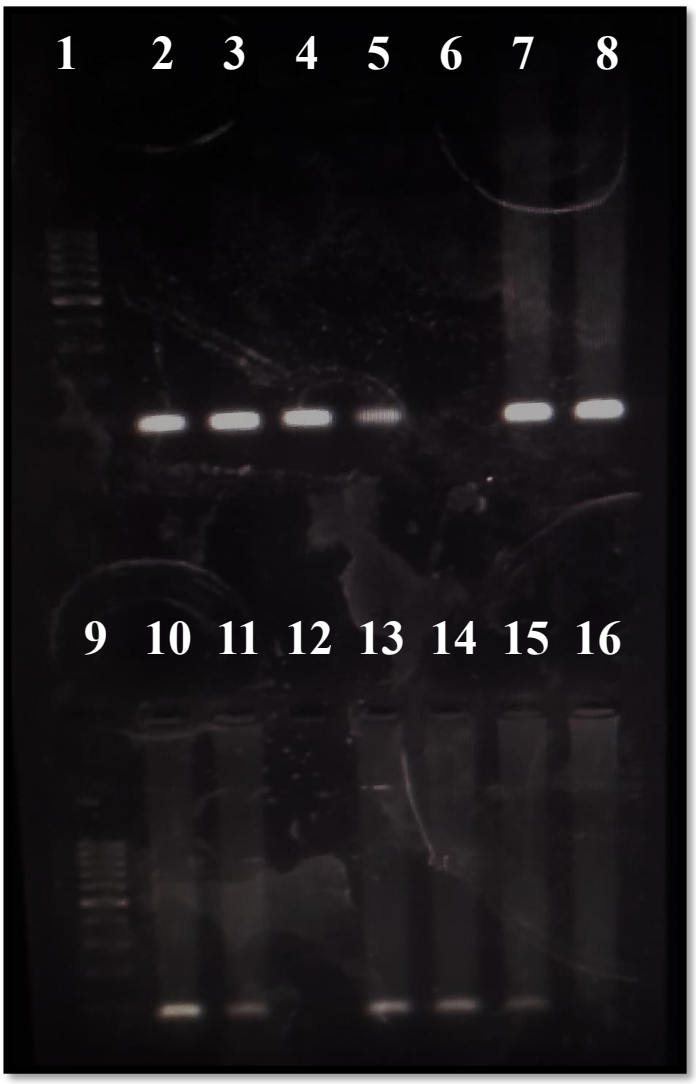


Plate 6. Agarose gel showing the 81 bp amplicons generated by conventional gradient PCR from primer set 5.

Lane 1 and 9: 100 bp ladder

Lane 2-5: Clinical sample (PCV2d) (Annealing temperatures 53, 55, 57 and 60℃ respectively)

Lane 7, 8, 10, 11: Clinical sample (PCV2b) (Annealing temperatures 53, 55, 57 and 60℃ respectively)

Lane 13-16: Clinical sample (PCV2h) (Annealing temperatures 53, 55, 57 and 60℃ respectively)

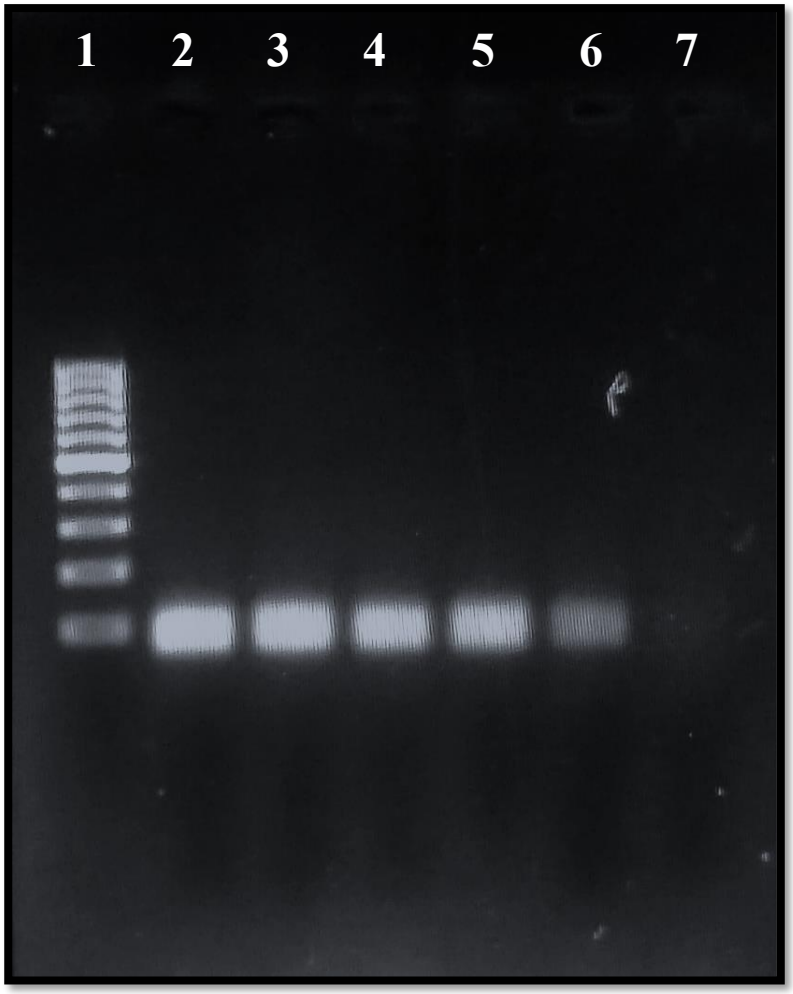


Plate 7. Agarose gel showing the 81 bp amplicons generated by conventional gradient PCR from primer set 6 for PCV2d.

Lane 1: 100 bp ladder

Lane 2: 52°C

Lane 3: 55°C

Lane 4: 56°C

Lane 5: 58°C

Lane 6: 60°C

Lane 7: 62°C

Table 11. Comparison of results of gel-based PCR and real-time PCR

Sl. No.	Sample no.	PCR	Ct Values	RT-PCR
1	33/MIB/2021	+	21.27	Positive
2	34/MIB/2021	+	30.36	Positive
3	47/MIB/2021	+	19.51	Positive
4	118/MIB/2021	+	39.12	Positive
5	122/MIB/2021	+	13.34	Positive
6	123/MIB/2021	+	24.4	Positive
7	124/MIB/2021	+	26.28	Positive
8	146/MIB/2021	+	33.3	Positive
9	168/MIB/2021	+	26.1	Positive
10	248/MIB/2021	+	39.14	Positive
11	312/MIB/2021	+	22.59	Positive
12	316/MIB/2021	+	19.98	Positive
13	370/MIB/2021	+	29.33	Positive
14	371/MIB/2021	+	29	Positive
15	432/MIB/2021	+	Undetermined	Negative
16	454/MIB/2021	+	37.35	Positive
17	45/MIB/2022	+	6.86	Positive
18	71/MIB/2022	+	24.82	Positive
19	169/MIB/2022	+	36.92	Positive
20	248/MIB/2022	+	37.21	Positive
21	132/MIB/2021	-	33.25	Positive
22	272/MIB/2021	-	33.22	Positive
23	273/MIB/2021	-	37.1	Positive
24	242/MIB/2022	-	27.43	Positive
25	16/MIB/2021	-	Undetermined	Negative
26	133/MIB/2021	-	Undetermined	Negative
27	181/MIB/2021	-	Undetermined	Negative
28	384/MIB/2021	-	Undetermined	Negative
29	394/MIB/2021	-	Undetermined	Negative
30	399/MIB/2021	-	Undetermined	Negative
31	427/MIB/2021	-	Undetermined	Negative
32	431/MIB/2021	-	Undetermined	Negative
33	437/MIB/2021	-	Undetermined	Negative
34	35/MIB/2022	-	Undetermined	Negative
35	40/MIB/2022	-	Undetermined	Negative
36	62/MIB/2022	-	Undetermined	Negative
37	88/MIB/2022	-	Undetermined	Negative
38	189/MIB/2022	-	Undetermined	Negative
39	194/MIB/2022	-	Undetermined	Negative
40	257/MIB/2022	-	Undetermined	Negative

Table 12. Comparison of results of gel-based PCR and real-time PCR

Conventional PCR	Real-time PCR	
	Positive	Negative
20 Positive	19	1
20 Negative	4	16

4.5.3 Determination of sensitivity of TaqMan real-time PCR based on copy number

The recombinant plasmid with PCV2 insert was obtained by performing cloning and it was confirmed by subjecting it to PCR. The sensitivity of TaqMan real-time PCR was estimated by using this plasmid.

4.5.3.1 Concentration of the recombinant plasmid

The copy number was determined using the recombinant plasmid DNA isolated by generation, gel purification, and cloning of the PCR products as described in 3.2.5.3.1. The concentration of the recombinant plasmid was estimated to be 6.18 ng/µL, using a Qubit 4 Fluorometer as described in 3.2.4.3.1.8.

4.5.3.2 Calculation of copy number

The copy number was calculated using the recombinant plasmid concentration by employing the formula mentioned in step 3.2.5.3.2. The copy number of the recombinant plasmid was estimated as 1.55×10^9 copies/µL.

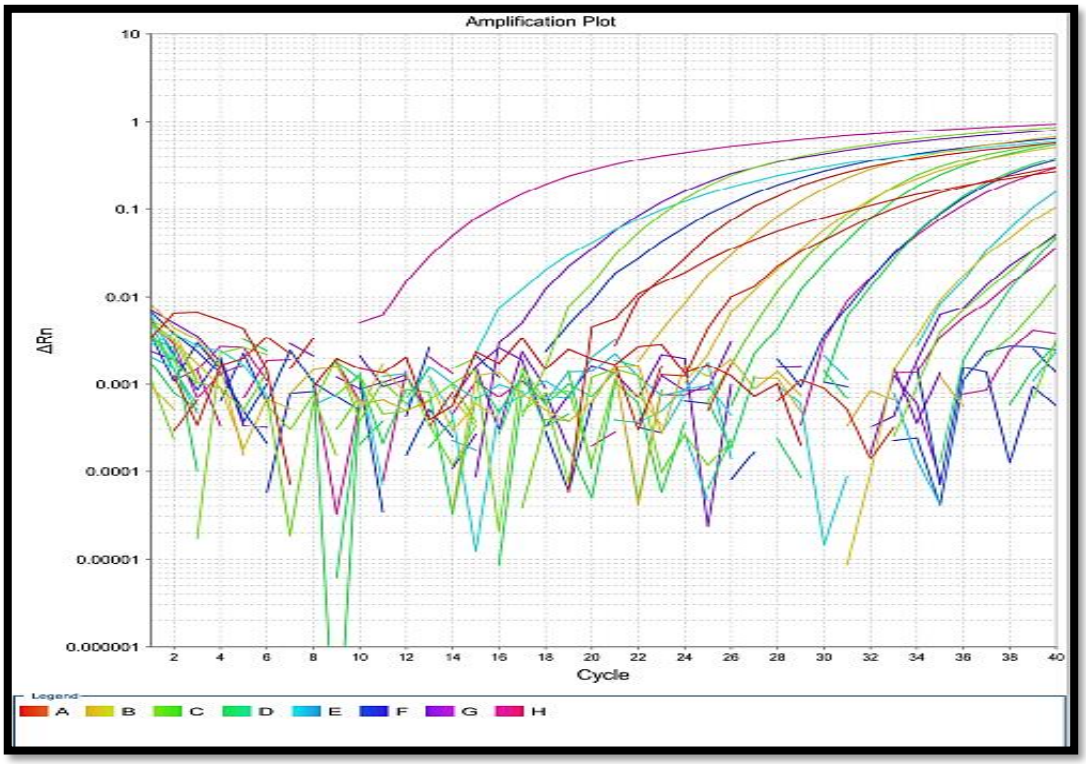
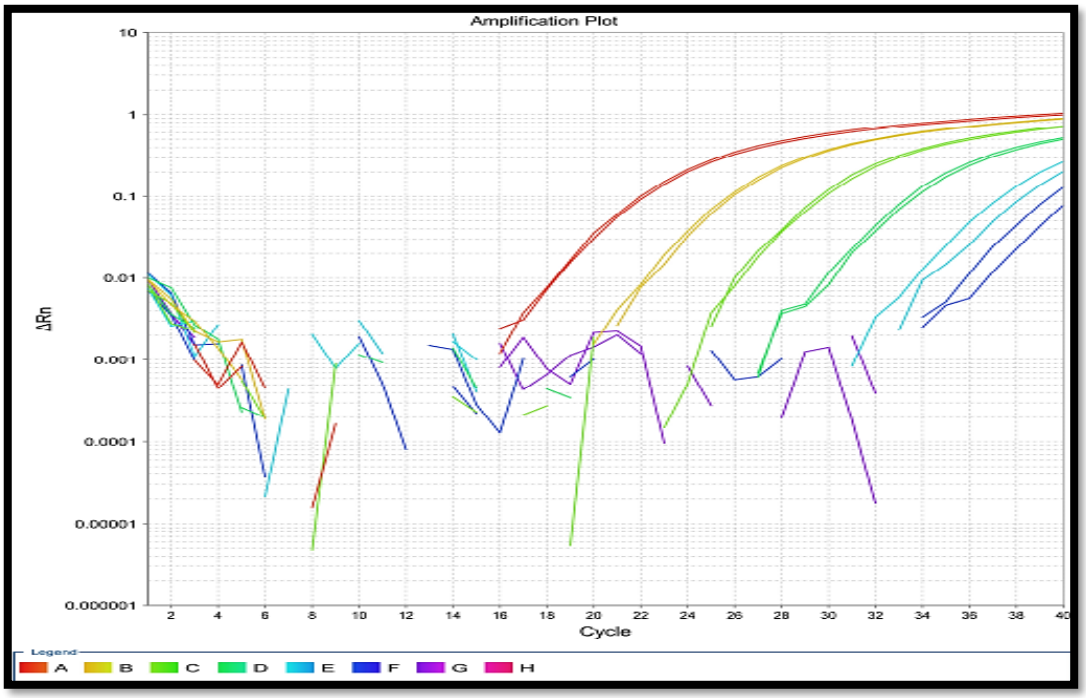


Plate 8. Amplification plot of clinical samples tested by TaqMan real-time PCR



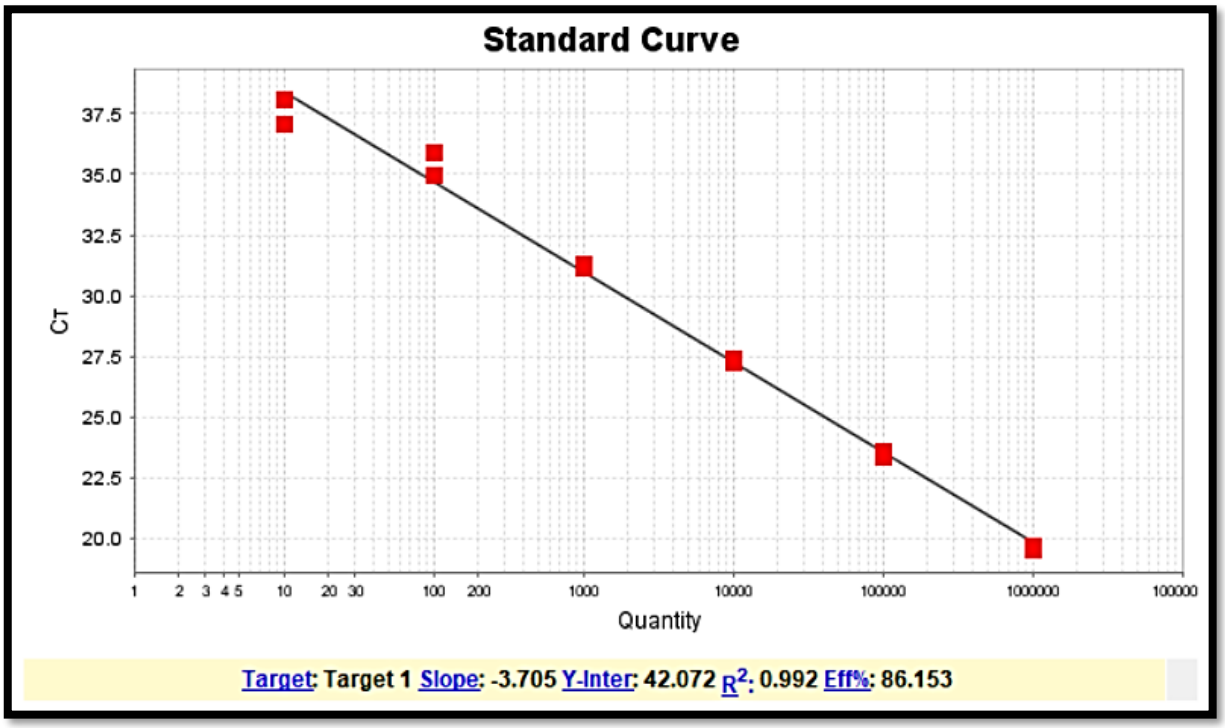


Plate 9. Determination of sensitivity of TaqMan real-time PCR in detecting PCV2 based on copy number

4.5.3.3 Sensitivity of the TaqMan real-time assay based on copy number

Serial 10-fold dilutions were made from 10^0 to 10^{-9} (corresponding to 1.55x 10^9 to 1.55x 10^0 respectively) in NFW along with 2 µL of the plasmid. TaqMan real-time PCR was carried out in duplicate using 2 µL of the plasmid by following the cycling conditions mentioned in step 3.2.5.2. Copy number was employed in calculating the sensitivity of the TaqMan real-time PCR. The highest dilution of the recombinant plasmid which demonstrated C_t value was considered as the endpoint. The endpoint obtained was 10^{-7} which corresponds to a copy number of 155 copies/µL and the detection limit of the assay was estimated to be 310 copies. The amplification plot and standard curve obtained are shown in Plate 9. Slope and R^2 were estimated to be -3.705 and 0.992, respectively. The efficiency of the TaqMan real-time PCR was calculated as 86.153 per cent.

4.5.3.4 Statistical analysis of the real-time assay using Kappa statistics

The sensitivity, specificity, and accuracy of the TaqMan real-time PCR were estimated to be 0.95, 0.80, and 0.875 respectively, in comparison with ORF2-based PCR. The percentage of agreement of the TaqMan real-time PCR based on ORF2 with conventional PCR based on ORF2 was analysed with the results obtained using Kappa statistics. Kappa or κ was estimated to be 0.75 which indicates substantial strength of agreement between the two tests.

Discussion

5. DISCUSSION

Due to the rapidly growing population, there is a rising demand for food. Consequently, the availability of healthy food is becoming a problem, necessitating an integrated approach to animal husbandry. Pigs have the highest feed conversion ratio of any livestock species, and hence pig farming has the highest potential in terms of meat production. It also provides profits in terms of manure and bristles along with meat. In India, pig farming provides a means of subsistence for rural poor from the lowest socioeconomic strata and offers seasonal rural employment opportunities. Disease occurrence in the farm can be deleterious to pig production as it leads to financial losses. Rapid and specific diagnosis of diseases is a key factor in the control of infectious diseases.

Subsequent to its initial description in the early 1990s, PCV2 has been linked to PCVAD, which has since become a serious illness that has a negative impact on the global economy (Allan and Ellis, 2000). In the recent past, PCV2 was considered an emerging threat to India's swine population (Parthiban *et al*., 2022). Most swine are exposed to PCV2 throughout their lifespan due to its ubiquity in nature which can cause subclinical and clinical infections. The absence of unique clinical symptoms and pathognomonic lesions in PCV2 infection makes it necessary to develop rapid and specific diagnostic tests. Though PCV2 was detected in Kerala in 2016 based on molecular methods, systematic characterization of the virus has not been carried out.

5.1 DETECTION OF PCV2

Chae (2004) reported that “diagnosis of PMWS must meet three criteria: (i) the presence of compatible clinical signs, (ii) the presence of characteristic microscopic lesions, and (iii) the presence of the PCV2 within these lesions”. The most accurate test for detecting PCV2 in clinical samples has reportedly been PCR (Calsamiglia *et al*. 2002; Grau-Roma *et al*. 2011; Segales, 2012).

5.1.1 Detection of PCV2 by conventional PCR based on ORF2

In the present study, PCR targeting the ORF2 gene was conducted to detect PCV2 in clinical samples. Sixty-two samples were screened for PCV2 and 36 samples were found positive. Percentage positivity was calculated as 58.06.

In Kerala, Aishwarya (2016) could detect PCV2 by PCR targeting the ORF2 gene region and found 5.26 per cent positivity among the samples tested. Also, in Kerala, Keerthana *et al*. (2017) could detect PCV2 by PCR and found 15.38 per cent positivity among the clinical samples tested. These are very much lower than what has been observed in this study indicating that the infection has spread in pigs in Kerala in the past few years. A prevalence rate of 11.56 per cent for PCV2 by ELISA, was reported by Pegu *et al*. (2017), in 1064 serum samples of pigs collected from North Eastern states of India and in the same study 86 tissue samples were, tested by PCR. In a similar study carried out by Mukherjee *et al.* (2018) in Meghalaya, a mean positivity of 62.25 per cent was reported for PCV2 after carrying out PCR. They also reported the highest antibody prevalence of PCV2 as 80.8 per cent in 2014, 79.1 per cent in 2015, and 96.2 per cent in 2016 in Meghalaya. A comprehensive study of PCV2 and its incidences in North East India from 2011 to 2017 indicated a 31.27 per cent mean positivity of PCV2 antibodies in the area (Barman *et al.*, 2018). Rajesh *et al*. (2019), reported a 49.35 per cent prevalence of PCV2 through ELISA in samples collected from all the North Eastern states of India. Parthiban *et al*. (2022) reported that the overall positivity percentage was 10.5 per cent with higher positivity in northern districts in comparison to the southern districts of Tamil Nadu, after carrying out specific PCR assay. Bhattacharjee *et al.* (2021) reported the PCR based PCV2 prevalence rate in Northeastern states of India, such as Tripura (20.8 per cent), Nagaland (25.0 per cent), Meghalaya (25.8 per cent), Assam (32.1 per cent), Sikkim (32.6 per cent), Manipur (33.3 per cent), Mizoram (36.7 per cent) and Arunachal Pradesh (42.3 per cent).

Kim *et al.* (2017) reported a 36.30 per cent prevalence of PCV2 in Korea after examining clinical samples by PCR. A PCV2 prevalence rate of 57.78 per cent in swine herds of Shanghai (Kang *et al.*, 2022) and 62.7 per cent in Henan province

of China (Zheng *et al.,* 2020) were reported after carrying out PCR. The PCV2 infection was detected in about 20 per cent of Hungarian wild boars (Cságola *et al*., 2006) and in Brazil, a high (54.09 to 89.53 per cent) prevalence was reported by Barbosa *et al*. (2006) by using PCR.

Several PCR methods have been described for the detection of PCV2 in fresh and fixed material (Allan *et al*., 1998, Kim and Chae, 2004, Larochelle *et al*., 1999, Mankertz *et al.*, 2000, West *et al*., 1999). Ellis *et al.* (1999) reported using PCR to detect PCV2 which targets a 481 bp fragment of the ORF 2 of the virus. Larochelle *et al*. (2003) could detect PCV2 based on primers targeting the ORF2 gene. They also opined that the test is suitable for detection as well as genetic characterization of PCV2 in clinical samples. In the present study also, PCR was found to be suitable for detection of the virus in clinical samples. As in this study, many other studies used tissues like lungs, lymph nodes, tonsils, and spleen for PCR (Larochelle *et al*., 1999; Fenaux *et al*., 2000; Calsamiglia *et al*., 2002).

5.2 TAQMAN REAL-TIME PCR ASSAY

The primers and probe for the TaqMan real-time PCR were designed from the ORF2 region of the aligned sequences (obtained ORF2 sequences and ORF2 sequences of all the Kerala isolates). The degeneracy was introduced in the forward primer (i.e., S= C/G) and the probe (i.e., M= A/C; W= A/T; V= A/C/G) to make them sensitive to all the genotypes of PCV2 reported in Kerala until now (Table 2, Set 5). However, it was observed that, though the primers could amplify the genotypes 2b, 2h and 2d, the probe did not detect these viruses and the test could not be standardized. Consequently, another forward primer and probe was designed to detect the 2d genotype as it was the most predominant genotype found in North Kerala (Table 2, Set 6). The detection limit of the assay was estimated to be 310 copies.

Out of 40 samples tested by TaqMan real-time PCR assay targeting the ORF2 gene region, 23 samples were found positive for PCV2. Percentage positivity was estimated to be 57.5, which is more when compared to that obtained in

ORF2-based PCR. This indicates that TaqMan real-time PCR assay is more sensitive than conventional PCR. When compared with ORF2-based conventional PCR, the TaqMan real-time PCR assay had a sensitivity, specificity, and accuracy of 0.826, 0.823, and 0.647 respectively. The estimated kappa value was 0.645 which indicated substantial agreement between the two tests. The increased sensitivity of the TaqMan real-time PCR obtained in this study is in agreement with the findings of Klein (2002) who reported that the TaqMan probe-based real-time PCR assay was more sensitive, accurate, and specific compared to the conventional PCR methods. The 5-3 exonuclease activity of the Taq DNA polymerase cleaves the dual-labelled fluorogenic probe of the TaqMan probe-based real-time PCR assay. The assay includes binding both primers and probes specific to their target sequences, eliminating the possibility of nonspecific amplification products and resulting in a more accurate result (Egli *et al*., 2001).

There are other reports also on the superior sensitivity of TaqMan Real-time PCR. Zhao *et al*. (2010) reported that on comparison of conventional PCR and TaqMan probe-based RT-PCR, the latter had the best detection rate for PCV-2. Additionally, TaqMan real-time PCR assay can be employed for detection of viral load and virus quantitation in tissues of pigs with PMWS and PDNS (Olvera *et al.*, 2004). Yuan *et al*. (2014) reported that the TaqMan real-time PCR was more sensitive than the conventional PCR assay.

The ORF2 of PCV2 has a lower nucleotide homology with porcine circovirus type 1 (PCV1) than ORF1 (~65%) (Liu *et al*., 2001), thus the Cap gene region (corresponding to nucleotides 1033–1734 bp of the whole PCV2 genome) is best suitable for designing primers and probes for detection of PCV2 (Olvera *et al.*, 2004).

5.3 MOLECULAR CHARACTERISATION OF PCV2 BY PCR

Samples that were positive for ORF2-based PCR were further subjected to PCRs for amplification of the complete genome of the virus using published primers and amplicons of 661 bp, 765 bp, and 676 bp were obtained which were sequenced. A total of eleven sequences were obtained and used for analysis.

5.3.1 Phylogenetic analysis of PCV2 based on ORF2

On BLAST analysis out of eleven sequences, eight sequences showed a high degree of homology with the other isolates from Kerala (MT828905, MW268738 and MW268739) (99.14-99.92 per cent identity) except isolates 78/MIB/2021 which had more identity with Uttar Pradesh isolate (MK035433) (99.26 per cent). The isolates 127/MIB/2021, and 128/MIB/2021 also had more identity with Uttar Pradesh isolate (MK035432) (99.00-99.04 per cent identity). Foreign isolates which showed a high percentage identity were of China (99.77-99.89 per cent identity) except isolates 33/MIB/2021, 127/MIB/2021, and 128/MIB/2021 which had more identity with Vietnam isolates (99.65-99.72 per cent identity).

A phylogenetic tree based on the complete ORF2 sequences of the 11 PCV2 strains sequenced here was constructed together with 62 reference sequences deposited in the GeneBank database presenting PCV2 genotypes from India, China, Vietnam, Malaysia, and South Korea. The phylogenetic tree grouped the eight samples into genotype 2d and cluster 13. Sample 78/MIB/2021 was grouped into genotype 2b and cluster 11, and samples 127/MIB/2021 and 128/MIB/2021 were grouped into genotype 2h and cluster 18. Among all 11 isolates, 1 comes under PCV2b (9.09 per cent), 8 belong to PCV2d (72.72 per cent), and 2 exist in PCV2h (18.18 per cent) indicating the predominant prevalence of PCV2d strain. A phylogenetic tree was constructed based on the partial ORF2 sequences previously submitted to GeneBank from Kerala, and sequences from India and abroad (Figure 3). Another phylogenetic tree was constructed using whole genomes (Figure 5). These two trees gave groups that were observed in the tree generated by using complete ORF2 sequences (Figure 4). The ORF2 region is considered the more

suitable phylogenetic and epidemiological marker for PCV2 and is able to reconstruct the same tree as the whole viral genome (Olvera *et al.,* 2007).

Larochelle *et al.* (2003) studied the phylogenetic relationship of the Canadian isolates of PCV2 based on ORF1 and ORF2 genes and reported that ORF2 had greater nucleotide variation than ORF1. Segales *et al.* (2008) classified PCV2 into four genotypes: PCV2a (earlier group II) with five subtypes or clusters (2A, 2B, 2C, 2D, and 2E), PCV2b (earlier group I) with three clusters (1A, 1B, and 1C), and PCV2C after the phylogenetic analysis based on the Cap gene and complete genome. Guo *et al.* (2010) reported PCV2d from China based on the phylogenetic analysis. Franzo and Segalés, (2018) revealed eight different genotypes after carrying out phylogenetic analysis of the ORF2 sequences of PCV2 namely, PCV2a, PCV2b, PCV2c, PCV2d, PCV2e, PCV2f, PCV2g, and PCV2h.

It was observed that prior to 2021, in North Kerala, only 2d genotype was detected (in 2015 Accession No. KX579945 and 2020, Accession No. MT828905, MW268738 and MW268739) (Figure 1) and this has been reported previously (Ravishankar *et al*., 2020). However, in 2021, 2b and 2h genotypes have been detected in North Kerala. It has been reported that in Central Kerala, the genotypes PCV2b and PCV2h are prevalent (Vijayaraghavan, 2021). In India, the genotypes 2a, 2b, 2d, 2f, 2g, and 2h, the clusters 2, 3, 10, 11, 13, and 18 of PCV2 are commonly detected (Bhattacharjee *et al.,* 2021). Also, Parthiban *et al.* (2022) reported that PCV2b, PCV2b-IM1, PCV2d, and PCV2d-2 genotypes are circulating in Tamil Nadu. Hence these genotypes would have been introduced to North Kerala thorough animal trade.

Franzo and Segales, (2018) reported that the prevalent genotypes were PCV2a in Africa and Oceania, PCV2b in America and Europe and PCV2d in Asia. Recent phylodynamic studies have revealed that the PCV-2 population dynamics have been characterised by the emergence of periodic waves of various genotypes that, after initially increasing in prevalence, spread along the main swine commercial routes before being replaced by subsequent emerging genotypes

(Franzo *et al*., 2016). Since its first report, PCV2a has been the predominate genotype. However, during 2003, PCV2b has become the predominate genotype associated with a number of clinical and subclinical infections, and reports of this global genetic shift from PCV2a to PCV2b have been made in many different geographic regions (Cortey *et al*., 2011, Franzo *et al*., 2015, Wang *et al*., 2020). From 2010 onwards, most of the global reports especially from Asia, revealed genotype shift from PCV2b to PCV2d (Xiao *et al*., 2015, Mukherjee *et al*., 2018; Yao *et al*., 2019) perhaps driven by the use of PCV-2 vaccines. These genotypic shifting associated with higher virulence and vaccine failures has raised the global concern for PCV2 infections (Karuppannan and Opriessnig, 2017).

5.4 AMINO ACID ANALYSIS

5.4.1 Analysis of amino acid encoded by ORF2

On analysis of the amino acid sequences of ORF2, it was observed that the sequences of 2d genotype were more or less conserved. 78/MIB/2021 was very close to the sequences from Mizoram, however, Mizoram sequences have a stop codon at the 234th position. 128/MIB/2021 was very close to the sequences from Uttar Pradesh and Tamil Nadu.

In the PCV2 capsid protein, four antibody recognition domains, A, B, C, and D, have been identified that are situated at positions 51 to 84, 113 to 132, 169 to 207, and 228 to 233, respectively (Khayat *et al.*, 2011). An alteration in the amino acid content at these locations may change the characteristics of the virus, including its virulence, tissue tropism, and immune response (Saha *et al*., 2012). In the present study, as far as Kerala isolates are concerned, position 169 (in domain C) showed the highest variation with Glycine (G), Serine (S), or Arginine (R) being present at this position. A unique finding of this study was the detection of Asparagine (N) at 234th position in isolate 118/MIB/2021. This change has not been recorded in Indian PCV2 so far.

The PCV2b-IM1 clusters identified in Tamil Nadu exhibited three distinct amino acid changes at residues 131 (T to P), 191 (AG to SA), and 232 (N/K to H), with extended amino acid lysine (K) at position 234 (Parthiban *et al*., 2022). Despite having similar amino acid changes at the same positions in the current study, isolate 128/MIB/2021 clusters with PCV2h/Cluster 18.

The Cap gene has showed several positions under positive selection and more amino acid changes than in the Rep gene (Olvera *et al*., 2007). The selective pressure on the Cap gene was of particular significance since it is the most exposed part of the virus and is the most likely to interact with the host immune system (Roitt and Male, 1998). Grenfell *et al*. (2004) reported that some viruses' epitopes will be under positive selection as a result of the intense immunologic pressure they are subjected to and they undergo rapid rate of amino acid modifications to thwart the immune response.

From the study it is concluded that three genotypes of PCV2 are prevalent in Kerala and that they are very similar to viruses reported from Tamil Nadu, Uttar Pradesh and Mizoram and that the 2d genotype predominates. These viruses might have been introduced into the State through animal trade. Due to the wide range of clinical implications that the virus can bring about in pigs, and the potential exacerbation of clinical signs due to coinfection with PRRS virus, the prevalence of the virus in pigs of Kerala is a cause for concern. Also, it was noticed that the TaqMan PCR was more sensitive than PCR for detection of PCV2. However, the attempt to develop a single assay for all the Kerala genotypes was not successful. Hence it would be prudent to use more than one molecular assay for detection of the virus in pigs in Kerala. Strict biosecurity measures, along with rapid and reliable testing should be implemented to control the spread of the virus thereby preventing economic losses to the pig farmers.

Summary

6. SUMMARY

Livestock farming plays a major role in economic development of India. A drastic decline in pig population was noticed from 2012 to 2019 and high incidence of infectious diseases may be one of the reasons for this. *Porcine circovirus 2* (PCV2) is an emerging swine pathogen causing enormous economic losses to the global swine industry and to the farmers. In Kerala, pig farming is mainly of semi-commercial type. Even though PCV2 has been reported in Kerala, no systematic study has been performed to characterise the virus and elucidate the lineage of the virus prevalent in pigs in Kerala. Diagnosis of PCV2 based on clinical signs is confusing because the symptoms of PCV2 overlap with that of many other infectious diseases like PRRS, PPV infection, swine influenza and *Mycoplasma hyopneumoniae.* So, there is a need for development of sensitive diagnostic tests for detection of PCV2 prevalent in Kerala. Hence, the present study was undertaken with the objectives of molecular characterisation of PCV2 in Kerala and development of a TaqMan probe based real-time PCR for detection of PCV2.

A total of 62 tissue samples were collected from pigs suspected to have died of for PCV2 infection and brought for post-mortem to the Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Pookode from various districts like Wayanad, Kozhikode, Kannur and Thrissur during 2021 and August 2022. For detection of PCV2, all samples were subjected to PCR based on ORF2 and in positive samples 481 bp amplicons were detected. Among 62 samples tested, 36 samples were positive (58.06 per cent) and 26 were negative (41.94 per cent). Eleven representative positive samples were subjected to PCR for amplification of the complete genome of the virus using published primers that amplified three overlapping genome fragments of size 661, 765, and 676 bp and these fragments were sequenced and whole genomes assembled.

On BLAST analysis of the whole genome sequences, it was found that all isolates belonged to PCV2. The isolates showed high percentage of identity to Indian isolates from Kerala and Uttar Pradesh and to isolates from China and

Vietnam. The complete genomes were submitted to GeneBank and the accession numbers were obtained.

Phylogenetic analysis based on partial ORF2, complete ORF2 and complete genome sequences clustered eight isolates under genotype 2d and cluster 13, two isolates under genotype 2h and cluster 18 and one isolate under genotype 2b and cluster 11 indicating the predominance of the former genotype in Kerala. It was observed that before 2021, only 2d genotype was prevalent in North Kerala, and that the 2b and 2h were introduced to the region in 2021, probably from Central Kerala or from other States where these genotypes were prevalent.

On amino acid analysis of ORF2 sequences, the isolates 47/MIB/2021 and 146/MIB/2021 had Glycine (G) at the 169th position (in domain C) were similar to some isolates from Tamil Nadu whereas Serine (S) and Arginine (R) was present in all the other isolates from Kerala and India. Also, the isolate 146/MIB/2021 had Glutamine (Q) at position 234 which was only present in isolates from Tamil Nadu. As far as Kerala sequences are concerned, position 169 showed the highest variation with Glycine (G), Serine (S), or Arginine (R) being present at that position. A unique finding of this study was the presence of Asparagine (N) at 234th position in one of the isolates. This change has not been recorded in Indian PCV2 so far.

Primers and probe for TaqMan real-time PCR targeting the ORF2 region were designed using the ORF2 sequences generated during the study and sequences of other Kerala and Indian isolates. To standardise the real-time PCR, gradient PCR was conducted using the designed primers with an annealing temperature range of 52℃ to 62℃. Though the designed primers were able to detect these genotypes, the probe was not able to detect these genotypes. Hence, a single TaqMan real-time PCR that could detect all the genotypes prevalent in Kerala could not be standardised. Therefore, another TaqMan assay specific for detection of PCV2d was designed as that genotype was predominant in North Kerala.

The ORF2 region of PCV2 was cloned using pJET1.2/blunt vector and the same was used for the determination of sensitivity of TaqMan real-time PCR. Serial

10-fold dilutions of the recombinant plasmid were made and the test was carried out. The detection limit of the assay was estimated to be 310 copies. The sensitivity, specificity and accuracy of the TaqMan real-time PCR were estimated to be 0.95, 0.80, and 0.875 respectively, in comparison with ORF2 based PCR. The estimated kappa value for comparison of ORF2 based conventional PCR and ORF2 based TaqMan real-time PCR was 0.75 which indicated that there is substantial strength of agreement between two tests.

A total of 40 samples (20 positive and 20 negative samples by ORF2 based conventional PCR) were tested by TaqMan real-time PCR based on ORF2 region. Among this, 23 samples were found to be positive by TaqMan assay. The percentage positivity of TaqMan assay (57.5 per cent) was more compared to ORF2 based conventional PCR (50 per cent). This indicates that, TaqMan is highly sensitive assay which could be used for the rapid diagnosis of PCV2.

The present study could detect the predominant prevalence of PCV2d in pig population of Kerala and PCR targeting ORF2 was found to be a reliable method for diagnosis of PCV2. This is the first report of molecular characterisation of PCV2 in Kerala based on partial and complete ORF2 and complete genome sequences and the study depicted the close similarity of the isolates with the other isolates of Kerala and with those from Mizoram, Uttar Pradesh and Tamil Nadu. The Kerala isolates were also closely related to the isolates from China and Vietnam. Unique amino acid change was noticed in one isolate which was not reported from India earlier. Also, a sensitive TaqMan real-time PCR based on ORF2 was developed and validated to be a sensitive assay for the detection of PCV2.

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APPENDIX

A) REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

i) Tris borate EDTA (TBE) buffer (5X)

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 mL

The pH of the stock solution was adjusted to approximately 8.3 and filtered through a 0.45 µm membrane filter. The working solution (0.5X) was made by diluting the stock solution as and when required.

ii) Ethidium bromide (1%)

Ethidium bromide (Sigma)	10 mg
Nuclease free water	1 mL

The solution was mixed and stored at 4°C. A concentration of 0.5 µg/mL was used in preparing agarose gel.

iii) Loading buffer (6X)

Bromophenol blue	0.25 g
Xylene cyanol FF	0.25 g
Sucrose	40 g
Distilled water to make up volume to	100 mL

B) REAGENTS FOR CLONING AND TRANSFORMATION

i) Ampicillin

Ampicillin (sodium salt)	100 mg
Distilled water to make volume to	1 mL

The powder was dissolved fully by mixing thoroughly and sterilized by filtration using 0.45 µm membrane filter and stored at -20°C.

ii) Luria-Bertini broth (LB broth)

LB broth powder	2.5 g
Distilled water to make volume to	1000 mL

Heated to dissolve powder and sterilized at 121°C for 15 min at 15 lbs/in^2 and stored at 4°C until used. Ampicillin was added at a final concentration of 100 µg/mL for selection of recombinants.

iii) Luria-Bertini agar (LB agar)

LB agar powder	4.0 g
Distilled water to make volume to	1000 mL

Heated to dissolve powder and sterilized at 121°C for 15 min at lbs/in^2. Cooled to around 46-48°C and added ampicillin at a final concentration of 1000 µg/mL for selection of recombinants and poured into sterile Petri plates and stored at 4°C until used.

MOLECULAR CHARACTERISATION OF *PORCINE CIRCOVIRUS 2* AND DEVELOPMENT OF A REAL-TIME POLYMERASE CHAIN REACTION FOR DETECTION OF THE VIRUS

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ABSTRACT

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KERALA, INDIA

ABSTRACT

Porcine circovirus 2 (PCV2) is an emerging swine pathogen causing enormous economic losses to the global swine industry. The virus has been reported in many of the pig-producing states of India including Kerala. However, no systematic study has been performed to characterize the virus and elucidate the lineage of the virus prevalent in pigs in Kerala. Hence, the present study was undertaken to characterise PCV2 in pigs in Kerala by molecular methods, and to develop a rapid and specific assay for the detection of the virus. A total of 62 tissue samples taken for the study were screened for the presence of PCV2 by ORF2-based PCR, in which 36 samples (58.06 per cent) were positive. The complete genome of the virus was sequenced from 11 representative positive samples. The sequences obtained revealed similarity to PCV2 sequences reported from India previously, and to sequences from China and Vietnam. Phylogenetic analysis was carried out based on complete genome, and partial and complete ORF2 sequences. The analysis revealed that of the 11 PCV2 genomes analysed, eight, two and one belonged to genotypes PCV2d, PCV2h, and PCV2b respectively. It was also noticed that prior to 2021, only genotype 2d was prevalent in North Kerala. Since 2021, 2b and 2h were detected in the region which may be due to introduction of these genotypes from other areas where these genotypes are prevalent. In case of analysis of ORF2 sequences, close clustering of Kerala sequences with those from Tamil Nadu, Uttar Pradesh and Mizoram were observed. The sequences were also similar to PCV2 sequences reported from Kerala previously. On analysis of the complete amino acid sequence of ORF2, it was observed that sample 118/MIB/2021 had Asparagine (N) at the 234th position, which was not been recorded in any Kerala or Indian sequences so far. Based on the ORF2 nucleotide sequences obtained in the study, primers and TaqMan probes were designed for the detection of PCV2 genotypes prevalent in Kerala. A single TaqMan real-time PCR that could detect all the genotypes could not be standardised. However, the designed primers (but not the probe) were able to detect these genotypes. Hence, another

TaqMan assay specific for detection of 2d was designed as that genotype was predominant in North Kerala. The detection limit estimated using the cloned template was found to be 310 copies of the viral genome. The designed TaqMan assay was used to test 40 samples (20 positive and 20 negative samples) and 23 samples (57.5 per cent) were positive indicating a higher sensitivity of the test.

Key words: PCV2, phylogeny, genotypes, PCR, TaqMan real-time PCR

Acad. Form No. 19

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY
Faculty of Veterinary and Animal Sciences
PROGRAMME OF RESEARCH WORK FOR THESIS FOR MASTERS DEGREE

1. Title of thesis

Molecular characterisation of *Porcine circovirus 2* and development of a real-time polymerase chain reaction for detection of the virus

2. a) Title of the department/ KVASU research project of which this forms a part

Nil

b) Code No. if any, and order by which the departmental/KVASU research project is approved

Not applicable

3. a) Name of student

Shashank. S.

b) Admission No.

20-MVP-36

c) Name of the discipline

Veterinary Microbiology

4. a) Name of Major advisor

Dr. Chintu Ravishankar

b) Address

Associate Professor
Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Pookode, Lakkidi P.O., Wayanad - 673 576

5. Objectives of the study

1. Molecular characterisation of *Porcine circovirus 2* (PCV-2) based on whole genome sequence of the virus
2. Development of a TaqMan probe based real-time polymerase chain reaction (RT-PCR) for detection of the virus

6. Practical/Scientific utility

Pig farming is a source of income for small and marginal farmers in Kerala. In recent years, PCV-2, along with porcine reproductive and respiratory syndrome virus, has become one of the major threats to pig industry resulting in enormous economic losses each year. The virus can cause a wide range of disease conditions in suckling and weaning pigs.

The disease is now prevalent in most of the pig producing states of India including Kerala. Only preliminary studies have been carried out to characterise the virus in Kerala. Hence, this study is undertaken to fully

characterise PCV-2 prevalent in Kerala by molecular methods and to develop a diagnostic test for rapid detection of the virus in clinical materials. This will help to understand the genotypes of PCV-2 in Kerala which will be invaluable for future vaccine development studies. Development of a rapid test for detection of the virus based on genome sequences of locally prevalent viruses will help to detect them efficiently so that control measures can be adopted quickly.

7. Important publications on which the study is based

The PCV-2 genome is circular single-stranded DNA with a size of 1.76 kb (Mankertz *et al*., 2000).

Zhao *et al*. (2010) reported that TaqMan probe based RT-PCR had the highest detection rate when compared to conventional PCR for detection of PCV-2.

Zhai *et al*. (2014) reported that the PCV-2 has three major open reading frames (ORFs) which are ORF1, ORF2 and ORF3.

Anoopraj *et al.* (2015) carried out the thorough investigation of genetic differences of PCV-2 isolates from India based on *cap* gene and complete genome sequence.

Bhattacharjee *et al.* (2015) reported the complete genome sequence of PCV2a and PCV2b from North Eastern states of India.

Aishwarya (2016) and Keerthana *et al*. (2017) reported the incidence of PCV-2 in pigs in Kerala.

Franzo and Segales (2018) reported a new genotyping scheme based on ORF2 sequences of PCV-2. In this scheme, eight genotypes of the virus, namely, PCV-2a to PCV-2h have been proposed.

Henriques *et al.* (2018) reported that the RT-PCR is the most simple and reliable method for the detection of PCV-2.

In Meghalaya, Mukherjee *et al.* (2018) employed PCR to test 190 tissue samples, collected during necropsy of pigs suspected to have died of PCV-2 infection, and found that 36 (18.94 per cent) were positive.

8. Outline of the technical programme

Tissue samples (tonsil, mesenteric lymph node, lung, liver and spleen) will be collected from a minimum of 40 pigs brought for post mortem to the Department of

Veterinary Pathology, College of Veterinary and Animal Sciences, Pookode. The samples will be collected only from pigs that have died exhibiting any of the symptoms of PCV-2 infection such as respiratory illness, gradual wasting, thriftiness, rough hair coat, polypnoea, dyspnoea, pallor, diarrhoea, icterus *etc*. The samples will be collected in sterile screw capped polypropylene vials using sterile scissors and forceps, and will be processed immediately or stored at -80°C until it is processed.

Total DNA will be extracted from the samples using standard procedure (Sambrook and Russell, 2001) or commercial kit. The DNA will be used as template in the PCR using previously published primers targeting ORF2 of PCV-2 (Ellis *et al.*, 1998) to detect the virus. The positive samples will be subjected to PCR for amplification of complete genome of the virus using published primers (Anoopraj *et al*., 2015). The obtained PCR amplicons will be sequenced. Phylogenetic analysis will be carried out using MEGAX software.

A TaqMan probe based RT-PCR will be standardised to detect the virus. The detection limit of the RT-PCR will be determined using cloned template. Sensitivity, specificity and accuracy of the RT-PCR compared to conventional PCR will be calculated.

9. Main items of observations to be made

1. PCR amplicon
2. Nucleotide sequence data
3. C_t value

10. Facilities

a) Existing

The work will be conducted with the existing facilities in the Department of Veterinary Microbiology and other departments of the College of Veterinary and Animal Sciences, Pookode.

b) Additional facilities required

Chemicals and biologicals

11. Duration of study

Four semesters

12. Financial estimate

Chemicals, biologicals, sequencing charges	: Rs. 24,000
Contingencies	: Rs. 1,000
Total	: Rs. 25,000

Signature of Student

Project co-ordination group proposed: Swine

Place: Pookode

Date: 22.06.2021

Signature of Major advisor

Name, address and signature of other members of Advisory Committee

1. Dr. Koshy John
   Professor and Head
   Department of Veterinary Microbiology,
   College of Veterinary and Animal Sciences, Pookode

2. Dr. Rajasekhar R.
   Assistant Professor
   Department of Veterinary Microbiology,
   College of Veterinary and Animal Sciences, Pookode

3. Dr. Anoopraj R.
   Assistant Professor
   Department of Veterinary Pathology,
   College of Veterinary and Animal Sciences, Pookode

13. References

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14. Time frame of work

Semester I

1. Collection of literature
2. Preparation of synopsis
3. Collection of samples

Semester II

1. Collection of samples
2. Testing of clinical samples using conventional PCR

Semester III

1. Collection of samples
2. Testing of clinical samples using conventional PCR
3. Sequencing of whole genome of PCV-2 in positive samples and analysis of sequences
4. Standardisation of TaqMan RT-PCR

Semester IV

1. Testing of clinical samples by TaqMan RT-PCR
2. Analysis of data
3. Compilation and preparation of thesis
4. Submission of thesis

CERTIFICATE

Certified that the research project has been formulated observing the stipulations laid down under the Prevention of Cruelty to Animals Act (Amendment, 1998).

Place: Pookode
Date: 22.06.2021

Dr. Chintu Ravishankar
Major Advisor

10. CURRICULUM VITAE

1.	Name of the candidate	:	Shashank S
2.	Date of birth	:	24-03-1998
3.	Place of birth	:	Mysuru
4.	Permanent address	:	Kallikoppal Village, Chikkadiganahalli Post, Gavadagere Hobli, Hunsur Taluk, Mysuru District. PIN-571610
5.	Major field of specialization	:	Veterinary Microbiology
6.	Educational qualification	:	B. V. Sc & A.H
7.	Professional experience	:	Nil

8. Academic achievements:
    - Abstract published in National and International conferences/seminars: 5
    - Papers published in journals/compendium: 5
    - Recipient of Kerala Veterinary and Animal Sciences University Junior Research Fellowship