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Pathological investigation and viral antigen distribution of postweaning multisystemic wasting syndrome in association with porcine parvovirus infection of pigs in Kerala, Southern India

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Abstract

Postweaning multisystemic wasting syndrome (PMWS) is one of the important emerging porcine circoviral associated disease (PCVAD) causing immense economic losses throughout the world producing mortality and morbidity mainly in post-weaned pigs. During the period from 2018-2019, a total of 50 pig carcasses from different parts of Kerala were screened for concurrent infections of porcine parvovirus (PPV) and porcine circovirus type 2 (PCV2), followed by the establishment of PMWS. All the samples were subjected to polymerase chain reaction (PCR) initially followed by histopathology and immunohistochemistry (IHC). Phylogenetic analysis was performed for PPV using non-structural gene -1(NS1). The result of the molecular screening using polymerase chain reaction revealed only one sample to be positive for both PCV2 and PPV. The

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gross lesions noted were the non-collapsed voluminous lungs, necrotising lymphadenitis, interstitial nephritis, splenomegaly and fibrinous pericarditis. Histopathological examination revealed mild interstitial pneumonia, lymphoid depletion in periarteriolar lymphoid sheaths of spleen with focal areas of granulomatous inflammation, depletion of the germinal centre of the follicles in the lymph nodes, non-suppurative myocarditis and interstitial nephritis. Further, immunohistochemistry demonstrated PCV2 and PPV antigens in the affected tissues. Based on the clinical signs, histopathological lesions and immunohistochemical demonstration of PCV2 antigen, the case was confirmed as PMWS in association with PPV. This report of PMWS in association with PPV confirms, probably for the first time in the Kerala state of India.

Keywords: *Post weaning multisystemic disease, porcine circovirus 2, porcine parvo virus, immunohistochemistry*

Porcine circovirus associated diseases (PCVAD) refers to several disease entities, associated with porcine circovirus type 2 (PCV2). Post-weaning multisystemic wasting syndrome (PMWS) is one of the most important PCVAD. Its first description was recorded at Canada in 1991 (Clark, 1997). Post-weaning mortality is the most significant problem in PMWS affected herds (Vijayaragavan *et al*., 2021). In addition, reduction in growth and poor feed conversion as well as increased usage of antibiotics added to the cost of this disease. Infection with PCV2 is mandatory for the PMWS to develop. Pathological studies of PCV2 infection with PMWS had only been recently established in Kerala (Keerthana *et al.,* 2017).

However, the most studies had shown that PCV2 needs one or more co-factors for PMWS to develop into severe and even fatal disease. Some of the identified co-factors were porcine parvo virus (PPV) (Ouyang *et al.,* 2019), porcine reproductive and respiratory syndrome virus (PRRSV) (Liu *et al.,* 2015), *Mycoplasma hyopneumoniae* (Opriessnig *et al.,* 2004). Among that, PPV have been demonstrated as one of the crucial co-factors in PMWS (Allan *et al.,* 1999). PPV is considered as a reproductive pathogen which is ubiquitous in nature and being the sole etiology of stillbirth, mummification, embryonic death and infertility (SMEDI) (Johnson and Collings, 1969). In India, PCV2 was first described in 2006 (Kumar, 2008) followed by identification of PPV in 2010 (Sharma and Saikumar, 2010), respectively. Concurrent infection with PPV increases the severity of PCV2 in post weaning multisystemic wasting syndrome (PMWS) (Kennedy *et al.,* 2000). But the interaction of these viruses to develop into PMWS has not been studied yet. To confirm, it must meet three criteria (i) the presence of compatible respiratory and digestive clinical signs (ii) characteristic histopathology lesions and (iii) demonstration of PCV2 antigen within the lesions. Hence, this study was designed with the objective of studying the pathological characteristics of natural cases of PMWS in association with PPV.

Materials and methods

The study was conducted for a period of one year from June 2018 to June 2019. The detailed history was noted followed by complete necropsy of the carcasses suspected of PMWS. Tissue samples such as lungs, lymph nodes, spleen, heart, liver, kidneys and tonsils of the soft palate were collected in 10 per cent neutral buffered formalin for histopathology and immunohistochemistry (IHC). The tissue samples were collected and immediately stored at -70 °C with proper labelling for molecular diagnosis.

Polymerase chain reaction

Total DNA was extracted from the pooled organ tissue samples for the

molecular screening of PCV2 and PPV using Qiagen DNeasy blood and tissue extraction kit (Germany) as per the manufacturer's recommendation. Polymerase chain reaction (PCR) was carried out for the screening of PCV2 and PPV. The forward and reverse primers used were 5′CGGATATTGTAGTCCTGGTCG3′; 5′ACTGTCAAGGCTACCACAGTCA3′ respectively targeting open reading frame – 2 (ORF-2) gene for screening PCV-2 (Ellis *et al*., 1999). The reaction was done in a 12.5 µL volume with 1µL each of primers with 10 *pM*/µL concentration. The target segment was 481 bp. The thermal cycling conditions included an initial denaturation of 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72°C for 10 min. For PPV detection, forward and reverse primer sequences used were 5′AGTTAGAATAGGATGCGAGGAA3′ and 5′AGAGTCTGTTGGTGTATTTATTGG3′ respectively targeting *NS1* gene of ORF-1 for screening PPV (Aishwarya *et al*., 2016). The target segment was 265 bp. The reaction was done in a 25 µL volume with 1µL each of primers with 10 *pM*/µL concentration. The thermal cycling conditions included an initial denaturation of 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 15 s, annealing at 53°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. Amplicons were analysed on 1.5 per cent agarose gel electrophoresis followed by visualisation in gel documentation system (Bio-Rad Laboratories, USA). Phylogenetic analysis of PPV sequences obtained in this study were carried out using MEGA7 software (Kumar *et al.,* 2016). For phylogenetic analysis, 21 sequences of PPV isolates from other parts of India and from other countries were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/). Using Clustal W program of MEGA7, alignment of the downloaded sequences followed by trimming of the same to match sequence lengths obtained in this study. The evolutionary history was inferred by using Neighbour Joining (NJ) method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the sequences analysed. Determination of evolutionary distances were done by Kimura 2-parameter method (Kimura, 1980), Jukes-Cantor parameter method (Jukes and Cantor, 1969) and Tamura-3-parameter method (Tamura, 1992).

Histopathology

Formalin fixed tissue samples were subjected through steps of dehydration, clearing, impregnation and paraffin embedding. The tissue sections were cut at 4-5 µm thickness and stained with haematoxylin and eosin (Suvarna *et al*., 2018).

Immunohistochemistry

The test was performed for PCV2 and PPV using PCV2 capsid antibody (GeneTex, USA, GTX128120) and PPV monoclonal antibody (Vmrd, USA, 3C9D11H11), respectively. Tissue sections were deparaffinised, rehydrated and citrate buffer method of antigen retrieval was used. The procedure was performed with avidin-biotin complex method as per the manufacturer's recommended protocol (Abcam secondary antibody kit, Cambridge, United Kingdom, Ab7090). 3' diaminobenzidine (DAB) chromogen was used as the colouring substrate for the positive antigenic signals. Mayer's haematoxylin was used as the counterstaining followed by mounting with DPX mountant.

Results and discussion

Molecular detection

A total of 50 randomly selected samples were tested for the presence of PCV2 and PPV. Screening showed only one case concurrently positive for both PCV2 and PPV.

The amplicons sizes of PCV2 and PPV were 481 bp (Fig.1) and 265 bp (Fig. 2), respectively. Kim and Chae (2004) proposed the PCR is the most sensitive technique compared to virus isolation, IHC and *in situ* hybridization in detecting PCV2 and PPV.

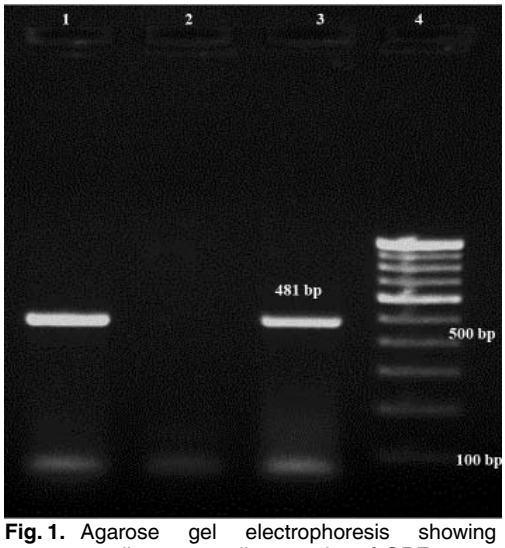


Fig. 1. Agarose gel electrophoresis showing amplicons encoding 481 bp of ORF2 gene of PCV2 from tissues using RT-PCR. Positive samples showed the expected molecular size of the 481 bp PCR product. Lane 1. Positive sample; Lane 2. Negative control; Lane 3. Positive sample; Lane 4.100 bp DNA molecular weight markers

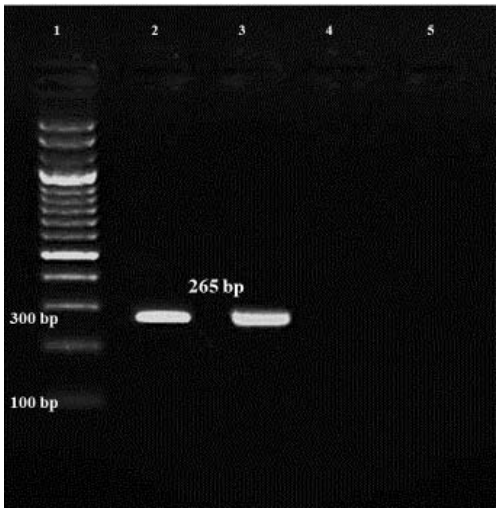


Fig. 2. Agarose gel electrophoresis showing amplicons encoding 265 bp of *NS1* gene of PPV from tissues using PCR. Positive samples showed the expected molecular size of the 265 bp PCR product. Lane 1.100 bp DNA molecular weight markers; Lane 2. Positive control; Lane 3. Positive sample; Lane 4. Negative sample

Pathological lesions

The lesions observed in PCV2 co-infected PPV pig were non-collapsed, voluminous lungs with rubbery consistency (Fig. 3). Lungs revealed frothy discharge with mucopurulent exudates on sectioning. Pleura was thickened and adhered to the thoracic cavity. Bronchial lymph nodes were enlarged, necrotic and haemorrhagic (Fig. 4). Fibrinous pericarditis with approximately 20-30 mL of blood-tinged fluid was observed in the pericardial sac (Fig. 5). Approximately 80-90

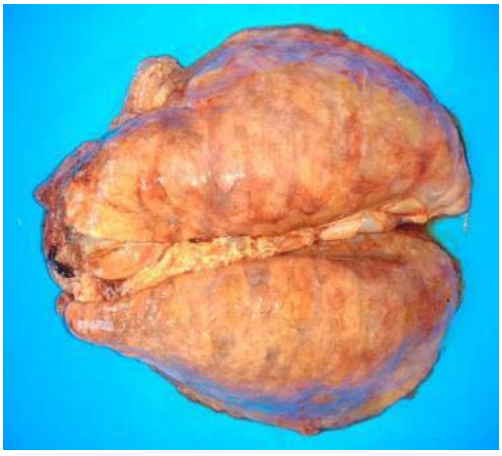


Fig. 3. Lungs: Non- collapsed voluminous lungs with rubbery consistency

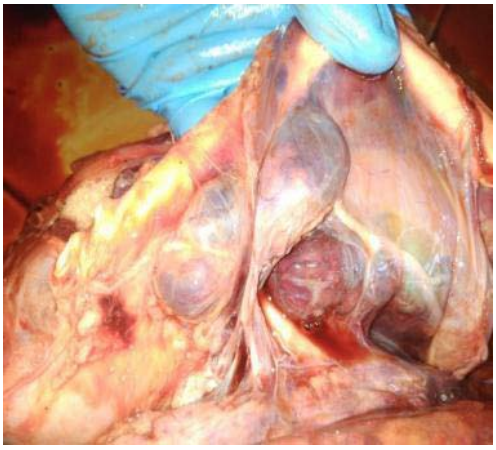


Fig. 4. Lungs: Enlarged, haemorrhagic and necrotic bronchial lymph nodes (arrow)

mL of serosanguinous fluid was also evident in the peritoneal cavity. Spleen was congested and enlarged. There were multifocal areas of interstitial nephritis in the kidney (Fig. 6). Liver showed multifocal white areas of necrosis (Fig. 7). Diphtheritic membrane was noticed in the mucosa of the large intestine. These lesions were also in accordance with the PCV2-SD categories of lesions as mentioned by Segales (2012). Woods *et al.* (2009) hypothesised that serosanguinous fluid in the peritoneal cavity was due to the outcome of endothelial damage by PPV. Drolet *et al.* (2002) demonstrated that positive co-relation exists between interstitial nephritis and association of PCV2 and PPV. The lesion was due to the ability of the virus to replicate in the dividing cells of tubular epithelium (Kennedy *et al.,* 2000).

Histopathological examination revealed mild broncho-interstitial pneumonia characterized by exfoliation of bronchiolar epithelium and thickening of alveolar septa due to proliferation of Type II pneumocytes with infiltration of mononuclear cells, haemorrhages, necrotic debris, and congestion of capillaries (Fig.8 and Fig. 9). Fibrin thrombi were noticed in the lumen of pulmonary blood vessels. There was

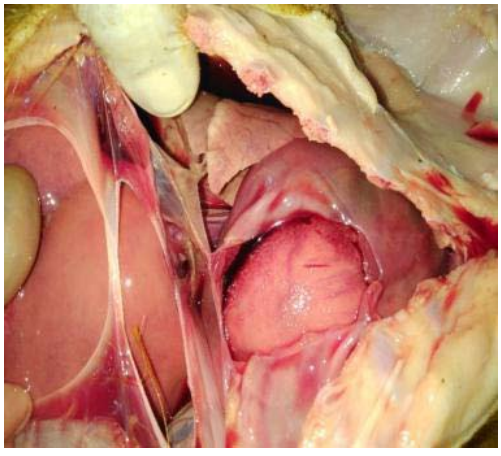


Fig. 5. Heart: Hemopericardium with fibrinous pericarditis (arrow)

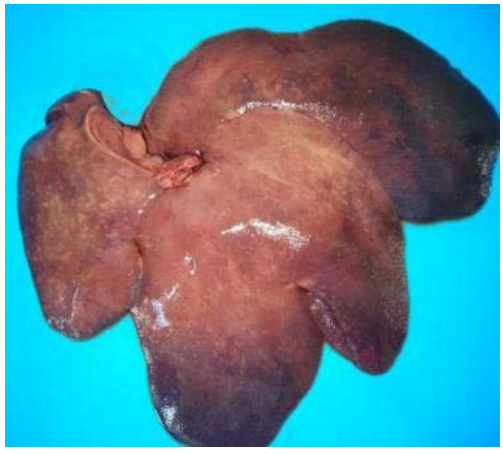


Fig. 7. Liver: Multifocal areas of necrotic foci

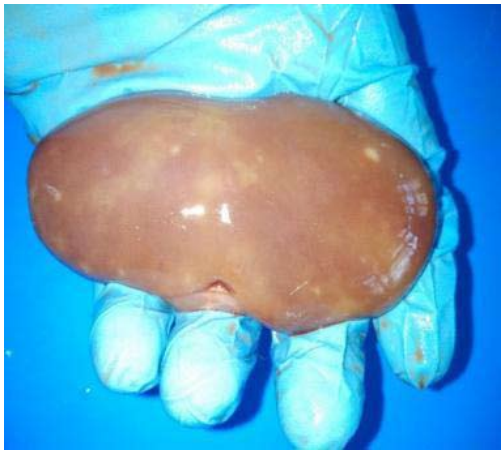


Fig. 6. Multifocal necrotic spots commonly called as "white spotted kidney' (arrows)

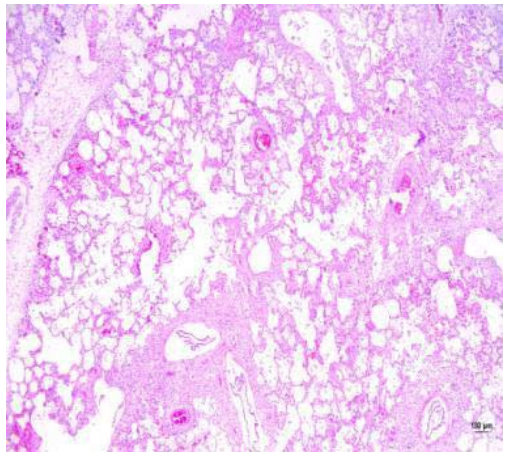


Fig. 8. Lungs (H&E x100): Mild broncho-interstitial pneumonia, proliferation of Type II pneumocytes with congestion of blood vessels

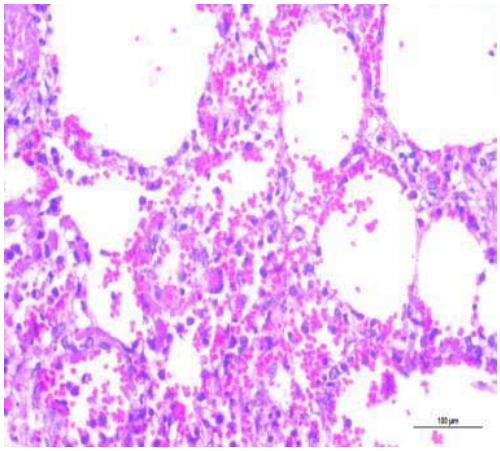


Fig. 9. Lungs (H&E x400): Infiltration of mononuclear cells, haemorrhages, necrotic debris, and congestion of capillaries

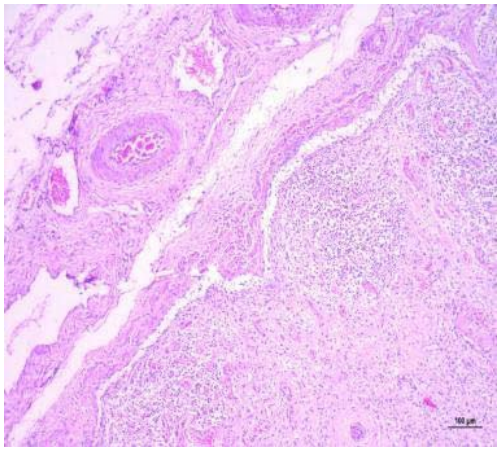


Fig. 10. Tonsil of the soft palate (H&E x100): Lymphoid rarefaction and necrosis with histiocytic infiltration

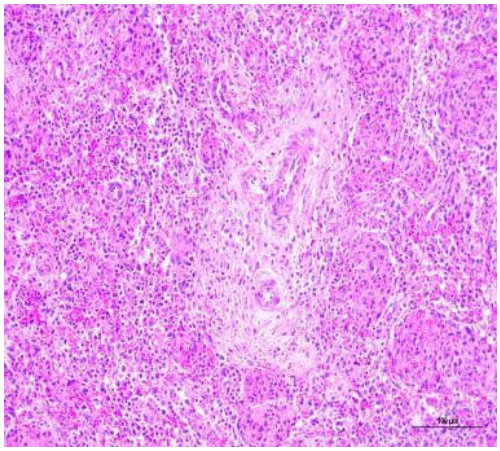


Fig. 11. Spleen (H&E x200) Lymphoid rarefaction of periarteriolar lymphoid sheaths (PALS) in the white pulp with infiltration of histiocytic and epithelioid cells

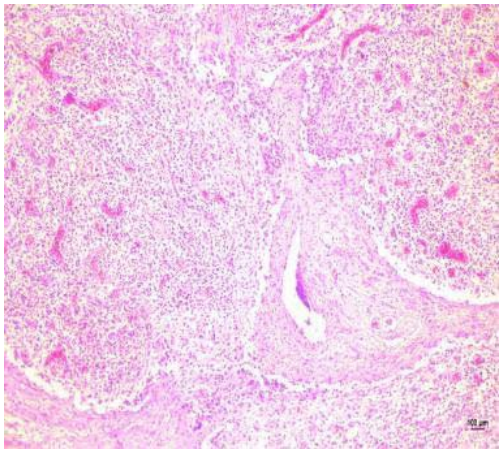


Fig. 12. Bronchial lymph nodes (H&E x100) Lymphoid depletion and congestion of blood vessels

moderate lymphoid rarefaction and necrosis with replacement by histiocytes in the tonsils of the soft palate (Fig. 10). Spleen revealed moderate rarefaction of periarteriolar lymphoid sheaths (PALS) in the white pulp region. Focal areas of granulomatous inflammation with the infiltration of epithelioid cells were also evident (Fig. 11). Bronchial lymph nodes showed depletion in the lymphoid density in the follicular areas with the infiltration of histiocytes and fewer eosinophils at the periphery of the follicles and congestion of blood vessels (Fig. 12). Non-suppurative myocarditis with infiltration of mononuclear cells and haemorrhages noticed between the cardiac muscle fibres. Vacuolar degeneration of the hepatocytes with focal areas of necrosis and infiltration of macrophages were seen in the liver. Kidney had multifocal areas of interstitial nephritis due to the infiltration of macrophages AND tubular necrosis and denuded epithelial cells was present in tubules (Fig. 13). There was also shrunken and variable sized glomerulus with increased periglomerular space in kidney. Histopathological lesions such as broncho-interstitial pneumonia with desquamated bronchiolar epithelium and proliferation of Type II pneumocytes with the infiltration of mononuclear cells was very

common findings noted in co-infection of PPV and PCV2 (Pescador *et al.,* 2007). Both PPV and PCV2 have high tropism to lymphoid cells which leads to impairment of immune system (Ellis *et al.,* 1998). So, enhanced replication of PCV2 resulted in the spread of PPV in lymphoid tissues (Allan *et al.,* 2000). These findings were similar to the findings reported by Ha *et al.* (2008). Non-suppurative myocarditis seen in the study was highly in consistent with PPV (Bolt *et al.,* 1997). They hypothesised that the porcine cardiac myocytes support the replication of PPV beyond the neonatal period.

Immunohistochemical demonstration of PCV2 and PPV antigens in tissues

Immunohistochemistry was performed to demonstrate PCV2 antigens mainly in the spleen and lymph nodes positive cases using PCV2 capsid antibody. Positive reaction was identified by typical dark brown reaction product with DAB as chromogen. Spleen showed maximum extent of positively stained cells predominantly in the inter-follicular region. The most consistent staining observed in this region was within lymphocytes followed by macrophages (Fig. 14). Lymph nodes had moderate to strong signals in the follicular region of cortex (Fig. 15). Here, moderate to strong signals was seen in lymphocytes followed by macrophages while in parafollicular region macrophages showed intense brown coloured staining than lymphocytes. Lymph nodes showed high extent of positive stained cells in comparison to spleen. Other cells such as reticuloendothelial cells and fibrocytes showed mild staining reaction. IHC was used for the demonstration of PPV antigens in tissues such as tonsil of the soft palate, lymph nodes and spleen. Strong immunopositivity was observed in the tonsil of the soft palate compared to other tissues. Signals were mainly observed in the follicles with lymphocytes, macrophages and

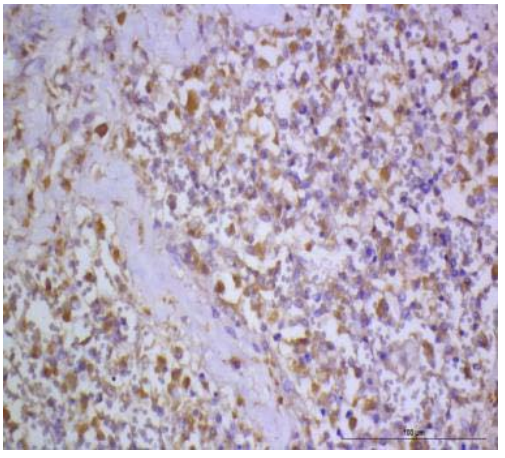


Fig. 14. Spleen (IHC x400): Positive PCV2 antigenic signals seen in the macrophages and lymphocytes in the interfollicular region

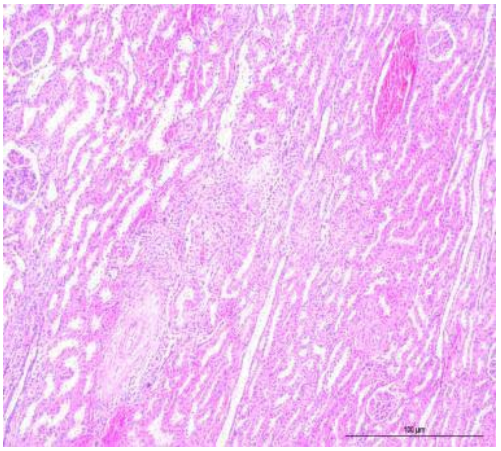


Fig. 13. Kidney (H&E x100): Multiple areas of tubular necrosis and interstitial nephritis (arrows)

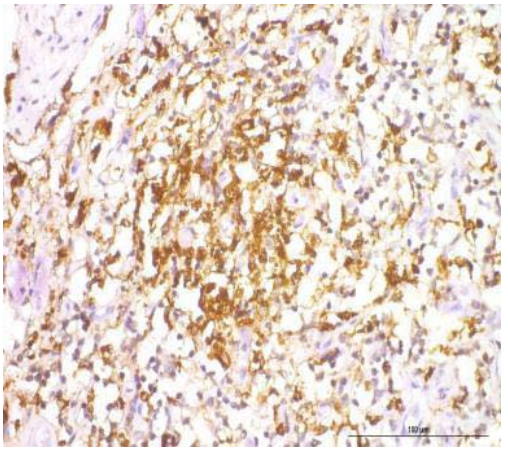


Fig. 15. Lymph nodes (IHC x400): Strong immunopositivity of PCV2 antigenic signals in the lymphocytes of the follicles in PPV positive case (arrow)

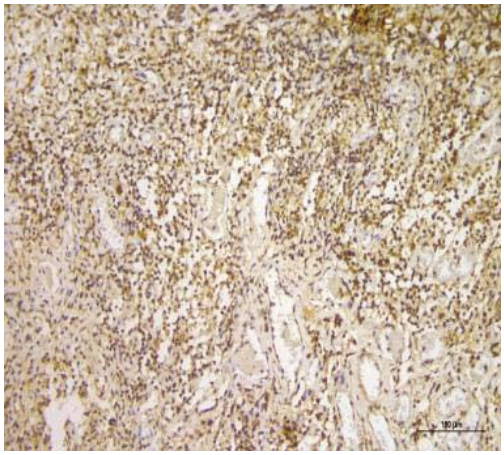


Fig. 16. Tonsils of the soft palate (IHC x100): Strong PPV positive signals in the cytoplasm of the macrophages, lymphocytes and few reticuloendothelial cells throughout the tonsils

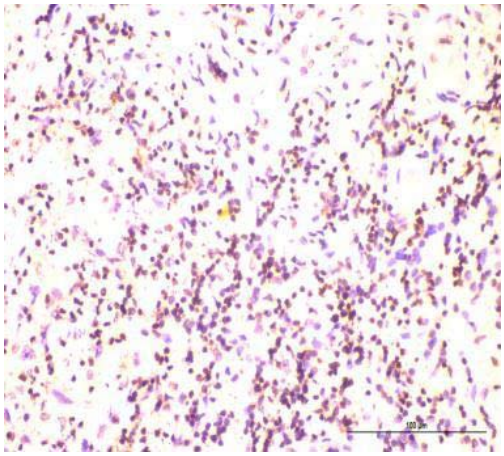


Fig. 17. Bronchial lymph nodes (IHC x200): Strong positive signals of PPV in the cytoplasm of infiltrated mononuclear cells and fewer lymphocytes (arrows)

reticuloendothelial cells (Fig. 16). Bronchial lymph nodes had positive signals of PPV in the cytoplasm of infiltrated macrophages, fewer lymphocytes and reticuloendothelial cells (Fig. 17). Spleen had positive immunolabelling mainly in the macrophages of the interfollicular areas and also noted in lymphocytes of cortex and paracortex areas. The PPV and PCV2 have high predilection to lymphoid cells such as macrophages and lymphocytes (Harding and Molitor, 1988). Positive PCV2 antigen could be demonstrated in follicular region of cortex and paracortex with a few reticuloendothelial cells in the lymph nodes. Spleen showed strong antigenic signals in the cytoplasm of the macrophages and some lymphocytes in the

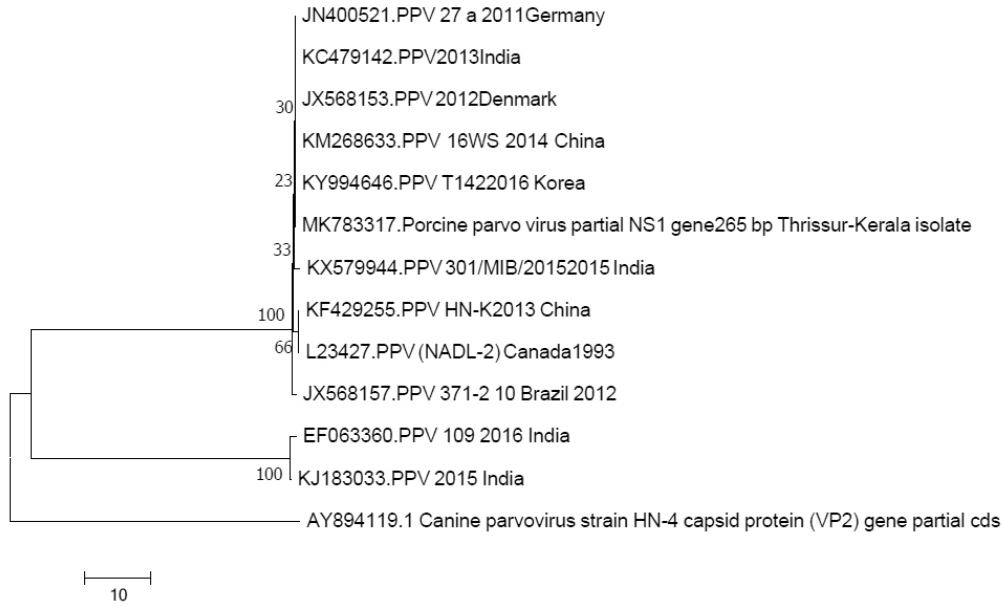
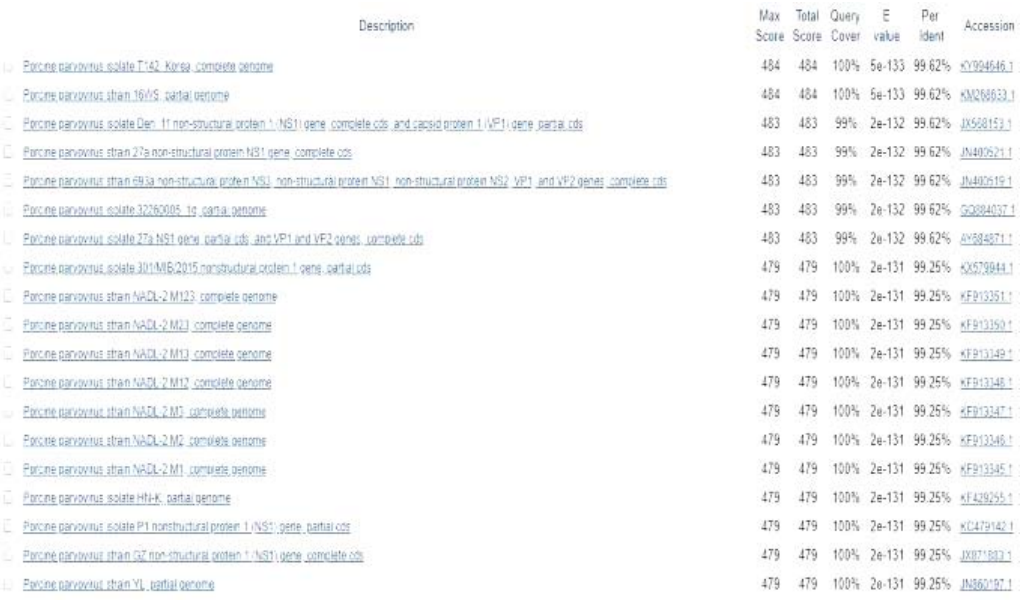


Fig. 18. Neighbour joining tree constructed from *NS1* gene sequences showing the phylogenetic relationships between PPV isolates. (Horizontal branch lengths are drawn to scale)



Description	Max Score	Total Score	Query Cover	E value	Per Ident	Accession
Porcine parvovirus isolate T142, Korea, complete genome	484	484	100%	5e-133	99.62%	KY994646.1
Porcine parvovirus strain 16/WS, partial genome	484	484	100%	5e-133	99.62%	KM268633.1
Porcine parvovirus isolate Den_11 non-structural protein 1 (NS1) gene, complete cds, and capsid protein 1 (VP1) gene, partial cds	483	483	99%	2e-132	99.62%	JX568153.1
Porcine parvovirus strain 27a non-structural protein NS1 gene, complete cds	483	483	99%	2e-132	99.62%	JN400521.1
Porcine parvovirus strain 6B3a non-structural protein NS3, non-structural protein NS1, non-structural protein NS2, VP1, and VP2 genes, complete cds	483	483	99%	2e-132	99.62%	JN400519.1
Porcine parvovirus isolate 32260085_1g, partial genome	483	483	99%	2e-132	99.62%	GQ884037.1
Porcine parvovirus isolate 27a NS1 gene, partial cds, and VP1 and VP2 genes, complete cds	483	483	99%	2e-132	99.62%	AY684871.1
Porcine parvovirus isolate 301/MIB/2015 nonstructural protein 1 gene, partial cds	479	479	100%	2e-131	99.25%	KX579944.1
Porcine parvovirus strain NADL-2 M123, complete genome	479	479	100%	2e-131	99.25%	KF913351.1
Porcine parvovirus strain NADL-2 M23, complete genome	479	479	100%	2e-131	99.25%	KF913350.1
Porcine parvovirus strain NADL-2 M13, complete genome	479	479	100%	2e-131	99.25%	KF913349.1
Porcine parvovirus strain NADL-2 M12, complete genome	479	479	100%	2e-131	99.25%	KF913348.1
Porcine parvovirus strain NADL-2 M3, complete genome	479	479	100%	2e-131	99.25%	KF913347.1
Porcine parvovirus strain NADL-2 M2, complete genome	479	479	100%	2e-131	99.25%	KF913346.1
Porcine parvovirus strain NADL-2 M1, complete genome	479	479	100%	2e-131	99.25%	KF913345.1
Porcine parvovirus isolate HN-K, partial genome	479	479	100%	2e-131	99.25%	KF429255.1
Porcine parvovirus isolate P1 nonstructural protein 1 (NS1) gene, partial cds	479	479	100%	2e-131	99.25%	KC479142.1
Porcine parvovirus strain GZ non-structural protein 1 (NS1) gene, complete cds	479	479	100%	2e-131	99.25%	JX871833.1
Porcine parvovirus strain YL, partial genome	479	479	100%	2e-131	99.25%	JN860197.1

Fig. 19. BLAST analysis of nucleotide sequence of the *NS1* gene of PPV

interfollicular region. The IHC results of PCV2 antigen were in agreement with the findings by Sairam *et al*. (2019). PPV positive antigenic signals were seen in the cytoplasm of infiltrated macrophages mainly in the interfollicular areas in the lymph nodes, lungs and spleen. Strong immunolabelling were observed in the interstitial macrophages. The results were in agreement with the findings by Kaur *et al.* (2016). Tonsils of the soft palate showed intense staining compared to other tissues such as spleen and lymph nodes which were similar with the findings of Kim and Chae (2004).

Analysis of NS1 gene sequences of PPV

Further characterisation was done by BLAST analysis. In the phylogenetic studies of PPV, NS1 having a fragment of 265 bp was taken for analysis. PPV isolate (Porcine parvo virus, partial NS1 gene, 265 bp, Thrissur-Kerala) was being sequenced during the study with the accession number MK783317 showed close relationship with the isolate PPV Korean isolate T142 (KY994646) and PPV isolate of Indian (Pookode) (301/MIB/2015) followed by isolates of Korea (KC800643), China (KM268633). The other Indian isolates of PPV namely porcine parvovirus clone HSADL India (KJ183033) from Bhopal formed a separate clade in the phylogenetic tree (Figs. 18 and 19). The PPV sequence isolated from the present study was closely related to the Indian and foreign isolates. Analysis of the sequence was done based on *NS1* gene which was considered as a conserved region (Hao *et al.,* 2011). Phylogenetic analysis of sequence of PPV isolate showed close relationship with Korean T142 (KY994646) followed by Indian isolate (Pookode) (KX579944), Chinese 16 WS (KM268633), Germany (AY684871) and Denmark (JX568153) isolates. More close relationship was shown towards the Korean strain than isolate of Pookode in this study. However, one of the published Indian isolates from HSADL, Bhopal, India (KJ183033) was slightly divergent and clustered in a separate branch in the phylogenetic tree. Therefore, close relationship of the sequence of PPV from this study with the Pookode isolate indicates the importance of circulation of isolate in

Kerala. Another route of entry of these isolates could be due to exotic pigs imported from other Asian and European countries for upgrading our native breeds. Nowadays, globalization in terms of interchange of animals and animal products was the prime factor for disseminating pathogens resulting in high risk of emergence of disease outbreaks (Cortey *et al.,* 2012).

Establishment of PMWS

The detection of PCV2 by PCR does not necessarily confirm the diagnosis of PMWS (Chae, 2004). The confirmation was performed using (i) the presence of compatible respiratory and digestive clinical signs (ii) characteristic histopathology lesions and (iii) demonstration of PCV2 antigen within the lesion. So, the cases were properly examined to meet the above-mentioned requirements. The clinical signs of PMWS were characterized by wasting, pallor of the skin, respiratory distress occasionally jaundice and diarrhoea were in agreement with the present study (Segales, 2012). Two main characteristic histopathological lesions of PMWS were granulomatous inflammation in lymphoid organs and intracytoplasmic botryoid inclusion bodies seen in the cytoplasm of the histiocytes. In this study, granulomatous inflammation was observed in the spleen but not the inclusion bodies. Kim *et al.* (2002) reported only 27.8 per cent of the PMWS cases examined had intracytoplasmic inclusion bodies, but 97 per cent of PMWS cases had granulomatous inflammation. Therefore, a more useful indicator in diagnosing PMWS is granulomatous inflammation. Finally, PCV2 antigen was demonstrated in the lesions using IHC in this study. IHC for the detection of PCV2 antigen in formalin fixed paraffin embedded tissue was considered more sensitive than *in situ* hybridization (McNeilly *et al.,* 1999). Therefore, the case of concurrent infection of PCV2 with PPV was confirmed as PMWS. Many co-factors known to increase the severity of PMWS have been identified. PPV is one of the important co-factors identified in association with PMWS noted much earlier by Ellis *et al.* (1999). Usually, PPV have been known in association with PCV2 reproductive disease (PCV2-RD) causing abortion and mummification (Sharma and Saikumar, 2010). But the present study demonstrated PPV related with PCV-SD resulting in mainly respiratory and digestive signs.

Conclusion

The PCV2 is an emerging swine pathogen causing immense economic losses in the global swine industry. Since the association with PMWS established in 1996, many studies have reported the pathological nature of this disease and its economic impact on commercial swine production. Since, many studies are now focussed on PMWS and its association with the other pathogens, this study has first time unveiled the association of PMWS with PPV in Kerala state. The limitation of the present study was that only one case fulfilled the requirement of PMWS and screening for secondary bacterial infection was not employed. Therefore, future studies should be directed to focus on screening for larger number of samples for PMWS and its interaction with different pathogens.

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Conflict of interest

The authors have no conflict of interest.

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