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Research Article

PATHOLOGICAL STUDIES ON NATURAL INFECTION OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS AND CO-INFECTION WITH PORCINE CIRCOVIRUS 2 ASSOCIATED POST-WEANING MULTI-SYSTEMIC WASTING SYNDROME AMONG SWINE HERDS OF KERALA, SOUTHERN INDIA

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ABSTRACT: Porcine reproductive and respiratory syndrome virus (PRRSV) infection is an endemic, economically important disease affecting the swine industry worldwide. Since its first outbreak in 2013, this disease has been rapidly spreading to different parts of India. The objectives of this study were to screen PRRSV among the swine population in Kerala, India, and to understand its pathological characteristics. Pathological investigation revealed lesions in respiratory and lymphoid organs, and viral antigen distribution was demonstrated using immunohistochemistry (IHC). Based on molecular screening using reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC), eight samples tested positive for PRRSV. These results were corroborated by gross and histopathological findings. Post-weaning multi-systemic wasting syndrome (PMWS) was also observed in two cases of PCV2-positive pigs co-infected with PRRSV. Additionally, the findings confirmed the mutual association of PRRSV and PCV-2 in PMWS among swine herds in southern India. The study identified the PRRSV strain as a Type II North American genotype (PRRSV-2). Further investigation of strains that are currently circulating in India is crucial to fully understand the diversity, distribution, and evolution of the virus.

Keywords: Immunohistochemistry, Molecular screening, PCV2, PMWS, PRRSV.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) infection is a major problem faced by the swine industry globally [1]. PRRSV is an enveloped, positive-sense, single-stranded RNA virus recently included in the genus Betarterivirus of the family Arteriviridae. Betarterivirus suid1 (formerly known as PRRSV-1) and Betarterivirus suid 2 (formerly known as PRRSV-2) [2] are the known viruses in this family. Clinically, PRRSV infection is characterized by respiratory disease and reproductive disorders in growing pigs and pregnant sows, respectively [3]. The major economic impacts of the disease include a reduction in the number of weaned piglets and an impairment of farrowing rates. In growing and finishing pigs, PRRSV can also result in mortality due to secondary bacterial infections, retarded growth and high antimicrobial usage [4]. Since the first outbreak in the United States in 1989 [5], the disease has been rapidly spreading throughout the world, including sporadic outbreaks in the North and North-Eastern parts of India [6]. The prevalence of the disease among the pig population of South Peninsular India is currently underestimated. Kerala is one of the leading

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swine-producing states in South India. According to the 2012 census report, the swine population of Kerala has shown a significant drop in the last 10 years [7]. Different factors could contribute to the loss of pig numbers in the state. Among those, diseases, especially due to viral etiologies, can cause drastic mortality in the swine population. In recent years, one of the major reasons for swine submissions to the Department of Veterinary Pathology was primary respiratory signs and reproductive illnesses. The major viruses involved in reproductive and respiratory failure in pigs that were previously detected in the state were PRRSV [8], porcine circovirus 2 (PCV2) [9], porcine parvovirus (PPV) [10], and classical swine fever virus (CSFV) [11]. Pathological studies of PCV2 infection have established its presence recently in Kerala [9]. There are many reports of PRRSV co-infection with one or more viral pathogens, including PCV-2, PPV and CSFV. Among these, PCV-2-induced post-weaning multisystemic wasting syndrome (PMWS) appears to aggravate the severity of the lesions of PRRSV infection [12]. PMWS is an endemic disease that has an enormous impact on the productivity of pig industries worldwide [13]. Clinically, PMWS is characterized by diarrhoea, wasting, dyspnoea, and pallor of the skin, with jaundice in the nursery and fattening pigs [14]. This study was designed to detect PRRSV infection and its pathology among the swine population of Kerala alongside recording common co-infections with PRRSV. Hence, the objectives focussed on the molecular detection of PRRSV, gross and histopathological characteristics of PRRSV infection, demonstration of viral antigens by immunohistochemistry (IHC), followed by sequencing and its phylogenetic analysis.

MATERIALS AND METHODS

Sample collection

Samples for molecular testing and histopathological evaluation were collected from 50 swine carcasses submitted to the Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy and Pookode, Kerala, from July 2018 to June 2019. The submitted swine carcasses were from 2 days to 1 year old at the time of death. Lungs, lymph nodes, tonsils of the soft palate, and spleen were collected in cryogenic vials with RNA later (Sigma Aldrich) and immediately stored at -70°C until further processing for molecular detection. Tissue samples from the lungs, lymph nodes, spleen and other organs with gross lesions were collected in 10% neutral buffered formalin for histopathology and IHC [15].

Molecular detection

Total RNA was extracted from the tissues stored in RNA later, using conventional methods of acid-guanidium-phenol-chloroform-isoamyl alcohol mixture as previously described [16]. The extracted RNA was eluted in 40 µL of RNAse-free water. Complementary DNA (cDNA) was synthesized from RNA using random hexamers utilizing the Revertaid H minus cDNA synthesis kit (Thermo Scientific, USA) according to the manufacturer's instructions and stored at -70°C. For the detection of PCV-2, DNA was extracted using the Qiagen DNeasy blood and tissue extraction kit (Hilden, Germany). Polymerase chain reaction (PCR) was performed with previously published primers and the thermocycling conditions listed in Table 1. The amplicons were visualized and documented using a gel documentation system (Bio-Rad Laboratories, USA).

Histopathology

Formalin-fixed tissues were cut at 4 µm thickness and stained with hematoxylin and eosin. The scoring of lesions in PRRSV-infected tissues was done semi-quantitatively as per previous literature [17]. The scoring patterns adopted in this study were: 0: tissue section that lacked the lesions; 1: a lesion that is minimal in severity and seen as 1 to 3 foci in the tissue section; 2: a lesion that is moderate in severity and multifocal in distribution; 3: severe and distributed multifocally with multiple confluent lesions; and 4: severe lesion and diffusely distributed in the tissue section. The Kruskal-Wallis test was used for statistical analysis of histopathological lesions of PRRSV infection in different tissues using SPSS software version 24.0 ($p < 0.05$).

Immunohistochemistry

An indirect IHC was performed for the detection of the M protein in PRRSV using a polyclonal antibody (GeneTex, USA, GTX129063). PCV2 capsid antibody was utilised to detect PCV2 antigen (GeneTex, USA, GTX128120). The antigen labeling reaction was demonstrated using the avidin-biotin complex technique (Abcam secondary antibody kit, Cambridge, United Kingdom, ab64264). Tissue sections were dewaxed and rehydrated and antigen retrieval was employed using citrate buffer at 95°C for 20 minutes, followed by blocking endogenous peroxidase with 3% hydrogen peroxide for 10 minutes. After washing with 1% Tris-buffered saline and Tween 20 (1% TBST), the protein block was carried out for 10 minutes. The sections were incubated at 4°C for 16 hours with a 1:250

dilution of the primary PRRSV M protein antibody in antibody dilution buffer. To identify PCV, a 1:200 dilution of PCV primary capsid antibody was applied to tissue sections and incubated at 4°C for 12 hours. The sections were washed with 1% TBST three times and flooded with biotinylated goat secondary antibody for 10 minutes, followed by subsequent washing with 1% TBST and treatment with streptavidin-horseradish peroxidase (HRP) for 3 minutes. The sections were rewashed with 1% TBST, and the 3'-diaminobenzidine (DAB) dye substrate was added and preserved for 3 minutes. Mayer's hematoxylin was applied as a counterstain for 5 minutes, followed by dehydration and DPX mounting. The scoring of IHC was performed semi-quantitatively based on the number of antigen-positive cells in the tissue section as follows: 0: virally negative tissue sections; 1: approximately 1 to 10 positive viral antigens in tissue sections; 2: tissue sections having approximately 11 to 30 positive viral antigens in tissue sections; 3: tissue sections containing approximately 31-100 positive viral antigenic signals in tissue sections; and 4: more than 100 positive viral antigens in the tissue sections [18]. Using version 24 of the SPSS software, the Kruskal-Wallis test was used to statistically analyze the immunohistochemical intensity of the PRRSV antigen in different tissues ($p<0.05$).

Nucleotide sequencing

The representative PCR products of the positive sample were sent to Agrigenome Lab Private Limited, Cochin, India, for sequencing of PRRSV. With the help of Chromas Lite v2.01 software (http://www.technelysium.com.au), the chromatograms of the sequences were analyzed. Blast analysis was conducted to confirm the presence of gene-specific PRRSV (http://www.ncbi.nlm.nih.gov/BLAST). Subsequently, sequences of other PRRSV isolates available in the GenBank database were procured and compared with those obtained in the present study.

Phylogenetic analysis

MEGA7 software was used to perform phylogenetic analysis on the sequence acquired in this study [19]. Twenty-one sequences of PRRSV isolates from India and other countries were downloaded from GenBank for phylogenetic analysis (www.ncbi.nlm.nih.gov/genbank/). Using the Clustal W program of MEGA7, alignment of the downloaded sequences was done, followed by trimming of the same to match the sequence lengths obtained in this study. The evolutionary history was inferred by using the Neighbour Joining (NJ) method [20]. The bootstrap consensus tree inferred from 1000 replicates [21] was taken to represent the evolutionary history of the sequences analyzed. Determination of evolutionary distances was done by the Kimura 2-parameter method [22], the Jukes-Cantor parameter method [23], and the Tamura 3-parameter method [24].

RESULTS AND DISCUSSION

Molecular detection

The PRRSV was tested in 50 swine carcasses used in this investigation employing RT-PCR that targeted ORF-6 (Fig. 8). Eight out of 50 samples were positive for PRRSV with an amplicon size of 451 bp (Fig. 1). Two of the eight PRRSV-positive samples were also positive for PCV2 with an amplicon size of 481 bp (Fig. 2). All 50 samples were negative for PPV and CSFV.

Pathological lesions

The most striking external lesion observed in two affected pigs was cutaneous erythema, especially in the ventral abdomen. Lungs appeared mottled, non-collapsed, and voluminous (Fig. 3A), with areas of petechial to ecchymotic hemorrhages. Diffuse pulmonary congestion and consolidation were observed with more prominent lesions in the cranio-ventral lungs, indicating possible broncho-interstitial pneumonia in six cases. At sectioning, frothy, blood-mixed fluid oozed out from the air passages of the lungs. The tonsils of the soft palate were moderately enlarged and hemorrhagic. Bronchial, prescapular, and mesenteric lymph nodes were severely enlarged and congested (Fig. 3B). Petechial to ecchymotic hemorrhages were noted diffusely in the heart, mainly in the left and right ventricular epicardium (Fig. 3C). Hemopericardium with 30-40 ml of blood-tinged fluid in the pericardial sac (Fig. 3D) and 40-50 ml of serosanguinous fluid in the peritoneal cavity was observed in five cases. Diffuse, white necrotic areas were seen throughout the liver (Fig. 3E). There was splenomegaly with randomly distributed areas of red infarction (Fig. 3F). A comprehensive histopathological examination of organs displaying gross lesions such as the lungs, tonsils of the soft palate, lymph nodes, spleen, kidney and liver, was performed in eight positive cases of PRRSV. Interstitial pneumonia was the predominant lung lesion when compared to broncho-interstitial pneumonia. Interstitial pneumonia was characterized by expanded alveolar septa due to the proliferation of type II pneumocytes

Table 1. Lists of primers used for detecting pathogens in this study.

Virus	Target region	Primer sequences (5'-3')	References
PRRSV	ORF-6	F - GAGTTTCAGCGGAACAATGG R- GCCGTTGACCGTAGTGGAG	[49]
PCV-2	ORF-2	F- CGGATATTGTAGTCCTGGTCG R- ACTGTCAAGGCTACCACGTA	[50]
PPV	NS1	F- AGTTAGAATAGGATGCGAGGAA R- AGAGTCTGTTGGTGTATTTATTGG	[10]
CSFV	E2	F- ATATATGCTCAAGGGCGAGT R- ACAGCAGTAGTATCCATTTCTTTA	[51]

[PRRSV: Porcine reproductive and respiratory syndrome virus, PCV-2: Porcine circovirus type 2, PPV: Porcine parvovirus, CSFV: Classical swine fever virus].

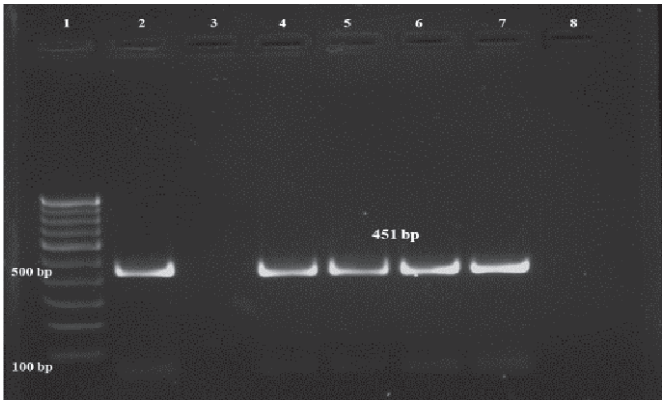

Fig. 1. Agarose gel electrophoresis showing PRRSV amplicon of 451 bp. DNA bands were visualized by staining the gel with ethidium bromide. [Lane 1: 100 bp DNA molecular weight markers; Lane 2: Positive control; Lane 3: Negative control; Lane 4-7: Test samples].

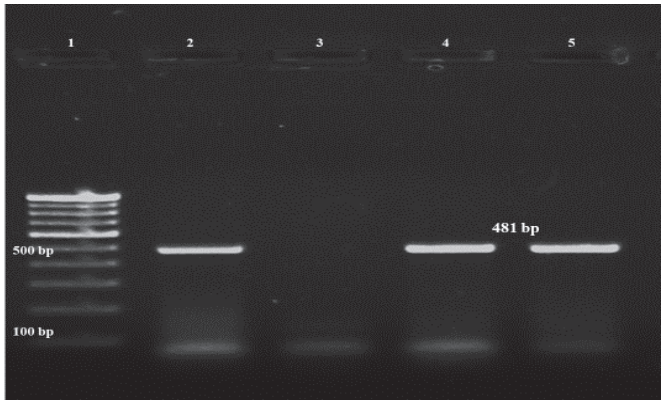

Fig. 2. Agarose gel electrophoresis showing PCV2 amplicon of 481 bp. [DNA bands were visualized by staining the gel with ethidium bromide. Lane 1: 100 bp DNA molecular weight markers; Lane 2: Positive control; Lane 3: Negative sample; Lane 4-5: Test samples].

and the infiltration of macrophages (Figs. 4A and 4B). Desquamation of the bronchiolar epithelium with sero-fibrinous exudates in the bronchiolar lumen and alveolar spaces and proliferation of type II pneumocytes were evident in broncho-interstitial pneumonia. In some cases, depletion of the bronchus-associated lymphoid tissue (BALT) was noticed. Follicular lymphoid hyperplasia was detected in the submucosa of the bronchioles. Lymph nodes revealed congestion and moderate-to-severe lymphoid depletion in the germinal center of the follicles (Fig. 4C). Lymphoid depletion was observed in the periarteriolar lymphoid sheath (PALS) of the spleen, with histiocyte infiltration at the periphery, fewer eosinophils and diffuse haemorrhages throughout the parenchyma. (Fig. 4D). Kidney revealed interstitial nephritis characterized by severe infiltration of mononuclear cells, predominantly macrophages, with tubular epithelial degeneration and necrosis. (Fig. 4E). Non-suppurative myocarditis with the infiltration of macrophages was detected. Severe degeneration of germinal cells with congestion of blood vessels was identified in the testicular parenchyma. Interstitial pneumonia and lymphoid depletion were exhaustive in co-infected cases of PRRSV with PCV-2 compared to PRRSV-infected cases alone. In lymph nodes, granulomatous inflammation was consistent with the presence of amorphous, small, intracytoplasmic botryoid inclusion bodies either single or numerous, which were seen among infiltrating macrophages (Figs. 4F and 4G). Focal areas of coagulative necrosis and infiltration of macrophages, plasma cells, eosinophils, and lymphocytes were observed in the sinusoidal space of the liver (Fig. 4H). Histopathological lesions revealed that among the tested tissue samples, lymph nodes had a high lesion score when compared to other tissues (Fig. 9).

Immunohistochemistry

The cells with PRRSV antigens appeared typically dark brown with DAB. Pulmonary alveolar and interstitial macrophages displayed moderate to strong

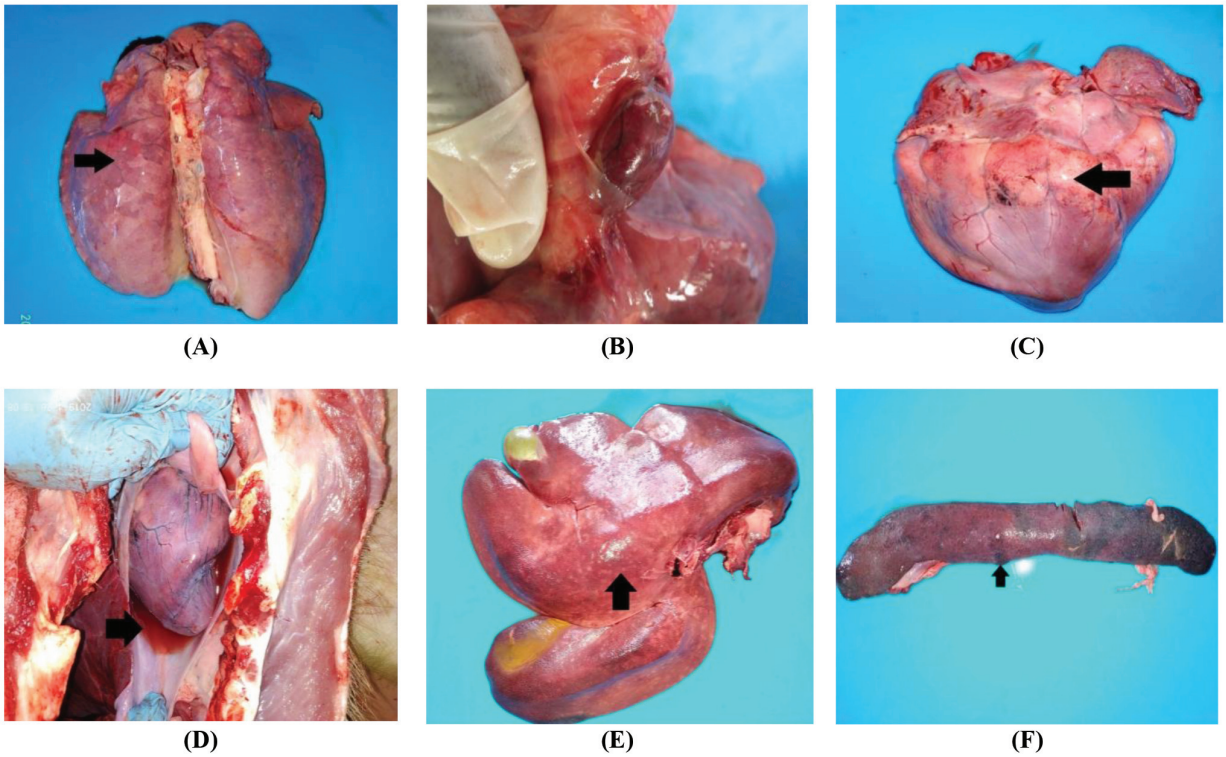

Fig. 3. Gross changes at post-mortem examination [A. Lungs: non-collapsed, voluminous lungs with mottling of interlobular septa (arrow); B. Bronchial lymph node: enlarged and congested; C. Heart: petechial haemorrhages randomly distributed on left and right ventricular epicardium (arrow); D. Heart: hemo-pericardium. Pericardial sac containing approximately 30-40 ml of blood-tinged fluid (arrow): E. Liver: multiple white areas of necrotic spots distributed throughout the visceral surface (arrow), F. splenomegaly with multiple areas of red infarction (arrow)].

positive signals (Fig. 5A). In a few lung tissues, moderate signals were noticed in the cytoplasm of the bronchiolar epithelium. Lymph nodes showed a greater extent of positively stained cells compared with the lungs. PRRSV antigens were demonstrated in the follicular region of the cortex, paracortex, and other reticuloendothelial cells. Positive signals were observed in the cytoplasm of the lymphocytes and macrophages/ histiocytes in the follicular region, while macrophages/ histiocytes mainly showed positive signals in the parafollicular region (Fig. 5B). The spleen showed the maximum extent of positively stained cells, predominantly in the inter-follicular region (Fig. 5C). The most frequently stained cells in this region were lymphocytes, followed by macrophages and occasionally fibrocytes. Cells with positive signals were readily detected in the cytoplasm of infiltrated macrophages and a few hepatocytes in liver. Infiltrated macrophages showed strong antigenic signals in the heart. Positive signals were detected in the tubular epithelial cells and infiltrating macrophages in the kidney. Positive PCV2 antigens were demonstrated in two cases of PRRSV. Strong PCV2 antigenic signals were mainly noted in lymphocytes of the germinal centers, whereas macrophages showed more antigenic signals in the inter-follicular region of lymph nodes (Fig. 5D). The spleen showed strong antigenic signals in the cytoplasm of lymphocytes and macrophages in the inter-follicular region. The IHC scoring revealed that among the tested tissue samples, lymph nodes had high antigen-positive cells when compared to other tissues (Fig. 9). However, there was no significant difference observed between IHC expression of PRRSV antigens in the lungs, lymph nodes and spleen ($p > 0.05$).

Sequencing and phylogenetic analysis

The MEGA7 software was used to perform the phylogenetic analysis of the generated sequences along with the sequences of PRRSV genes from India and other countries that were downloaded from GenBank. For the ORF6 gene sequences, a 451 bp fragment was taken for the phylogenetic analysis. The PRRSV isolate was sequenced during the study with accession number MK882922 (PRRSV Thrissur-Kerala isolate, India 2019). Blast analysis of the ORF6 sequence obtained in this study revealed a high degree of homology with

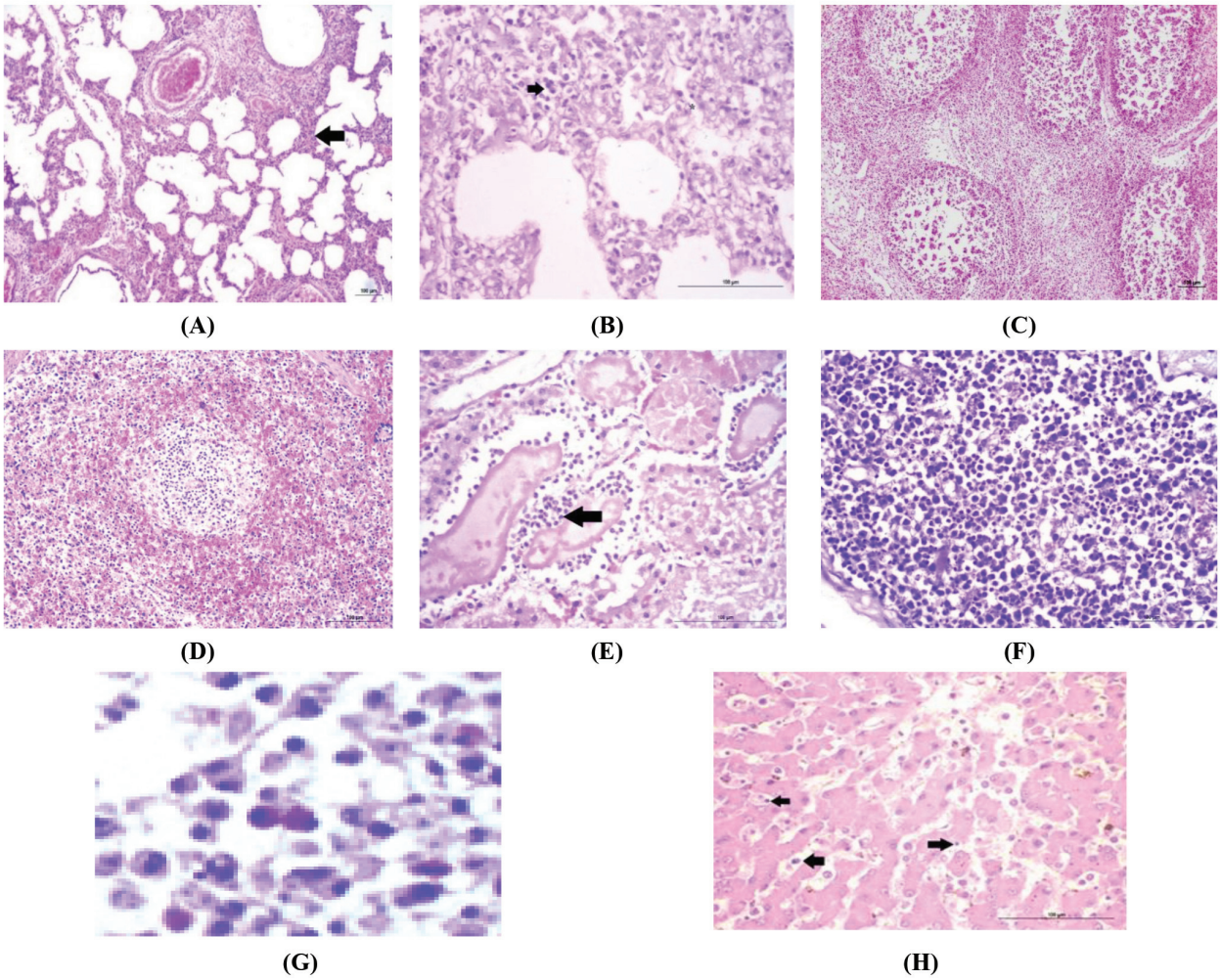

Fig. 4. Histopathological examination. [A. Lungs: Interstitial pneumonia characterized by expanded alveolar septa due to the proliferation of Type II pneumocytes with infiltration of mononuclear cells leading to reduced alveolar spaces. H&E x100. B. Lungs: Thickened alveolar septa due to the proliferation of Type II alveolar cells and infiltration of the mononuclear cells in the alveolar interstitium (arrow), H&E x400. C. Lymph nodes: Severe depletion of lymphoid follicles, H&E x200. D. Spleen: Severe depletion of periarteriolar lymphoid sheath (PALS), H&E x200. E. Kidney: Interstitial nephritis with infiltration of mononuclear cells (arrow) and tubular epithelial degeneration and necrosis, H&E x400. F. Lymph nodes: Granulomatous inflammation characterised by the severe infiltration of epithelioid cells in co-infected cases of PRRSV with PCV2, H&E X400. G. High power of Fig. 4F. Small, intracytoplasmic, amorphous, botryoid inclusion bodies were noticed in macrophages. H&E x1000. H. Liver: Coagulative necrosis of the hepatocytes and infiltration of macrophages, plasma cells and lymphocytes (arrow), H&E x400].

isolates from India and China (JF268672, EU563949, MF370557, KP771781, KP771777) (96.90%-99.08% identity). Assam isolates had a 99.08% identity, while Chinese isolates had a higher identity between 96.90% and 97.12% with the study isolate. The result of the blast analysis is given in Fig. 6. The alignment of the isolate sequences with the PRRSV Indian and international reference isolates indicated that the isolate is genetically linked to other Indian isolates but belongs to a separate clade (Fig. 7). Based on the analysis of the phylogenetic tree, the study isolate is not closely related to the other Indian isolates.

We studied swine carcasses to detect the PRRSV infection, the most important emerging viral pathogen, in Kerala, India. Clinical signs with a supportive history and gross lesions suggested the high possibility of PRRSV infection. Additionally, we investigated other viruses involved in reproductive and respiratory disorders, such as PCV-2, PPV, and CSFV. Overall, eight PRRSV-positive cases were detected based on RT-PCR and IHC. Eight samples yielded an amplicon size of 451 bp and were determined to be positive for PRRSV. RT-PCR is one of the most reliable strategies for pathogen detection when compared to virus

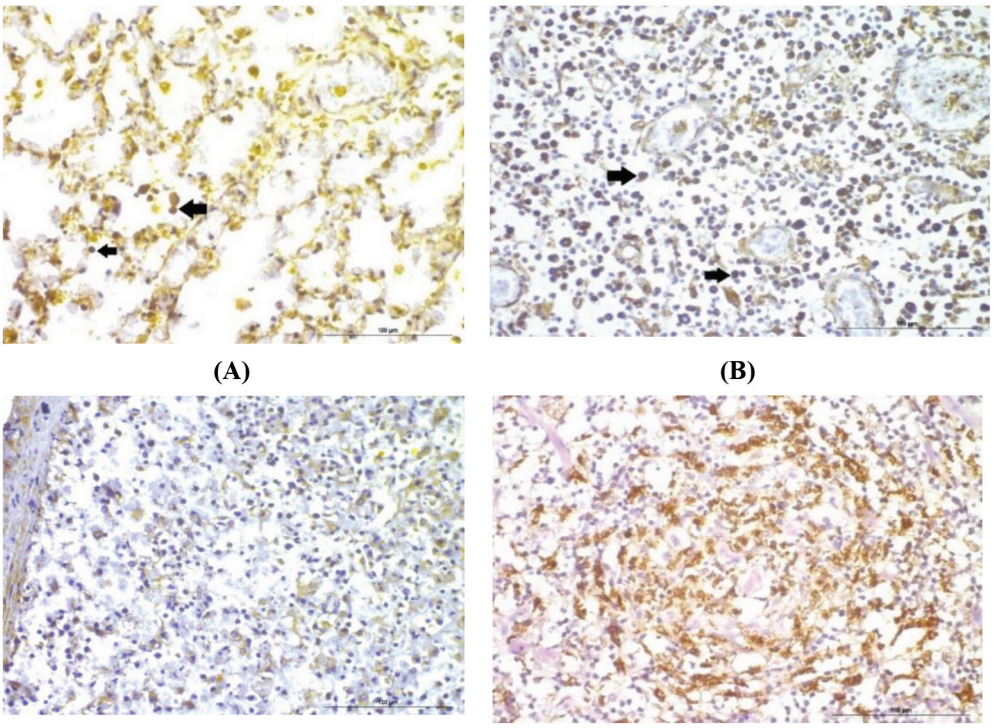

Fig. 5. Demonstration of viral antigens by immunohistochemistry. [A. Lungs: Intensive expression of PRRSV antigens in infiltrated macrophages and in a few pneumocytes (arrows). B. Lymph nodes: Positive PRRSV signals in the cytoplasm of macrophages and lymphocytes (arrows). C. Spleen: Positive PRRSV signals in the cytoplasm of the infiltrated macrophages and lymphocytes. D: Lymph nodes: Strong immunopositivity of PCV2 antigenic signals in the lymphocytes of the follicles].

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Porcine reproductive and respiratory syndrome virus strain 09SC, complete genome	761	761	100%	0.0	97.12%	JF268672.1
Porcine respiratory and reproductive syndrome virus strain HBGS GP2a, GP2b, GP3, GP4, GP5, M matrix protein, and N nucleocapsid protein genes, complete cds	761	761	100%	0.0	97.12%	EU563949.1
Porcine reproductive and respiratory syndrome virus strain FZ06A, complete genome	756	756	100%	0.0	96.90%	MF370557.1
Porcine reproductive and respiratory syndrome virus strain NVDC-HuNCS-2014, complete genome	756	756	100%	0.0	96.90%	KP771781.1
Porcine reproductive and respiratory syndrome virus strain NVDC-CQ4-2012, complete genome	756	756	100%	0.0	96.90%	KP771777.1
Porcine reproductive and respiratory syndrome virus strain NVDC-CQ2-2012, complete genome	756	756	100%	0.0	96.90%	KP771776.1
Porcine reproductive and respiratory syndrome virus strain NVDC-SD2-2012, complete genome	756	756	100%	0.0	96.90%	KP771768.1
Porcine reproductive and respiratory syndrome virus strain NVDC-CQ1-2012, complete genome	756	756	100%	0.0	96.90%	KP771747.1
Porcine reproductive and respiratory syndrome virus strain 10ZQ-GD, complete genome	756	756	100%	0.0	96.90%	JX192639.1
Porcine reproductive and respiratory syndrome virus strain HV, complete genome	756	756	100%	0.0	96.90%	JX317648.1
Porcine reproductive and respiratory syndrome virus strain GDQY1, complete genome	756	756	100%	0.0	96.90%	JN387271.1
Porcine reproductive and respiratory syndrome virus strain 09HUB1, complete genome	756	756	100%	0.0	96.90%	JF268682.1
Porcine reproductive and respiratory syndrome virus strain 09HEN2, complete genome	756	756	100%	0.0	96.90%	JF268680.1
Porcine reproductive and respiratory syndrome virus strain BJSY07, complete genome	756	756	100%	0.0	96.90%	HM011104.1
Porcine reproductive and respiratory syndrome virus strain YN9, complete genome	756	756	100%	0.0	96.90%	GU232738.1
Porcine respiratory and reproductive syndrome virus strain BJBLZ, complete genome	756	756	100%	0.0	96.90%	FJ950745.1
Porcine respiratory and reproductive syndrome virus strain BJSY-1, complete genome	756	756	100%	0.0	96.90%	FJ950744.1
Porcine respiratory and reproductive syndrome virus strain CWZ-1-F3, complete genome	756	756	100%	0.0	96.90%	FJ889130.1
Porcine respiratory and reproductive syndrome virus strain CBB-1-F3, complete genome	756	756	100%	0.0	96.90%	FJ889129.1
Porcine respiratory and reproductive syndrome virus strain SX2009, complete genome	756	756	100%	0.0	96.90%	FJ895329.1
Porcine respiratory and reproductive syndrome virus strain 07BJ, complete genome	756	756	100%	0.0	96.90%	FJ393459.1

Fig. 6. BLAST analysis of nucleotide sequence of the ORF6 gene of PRRSV.

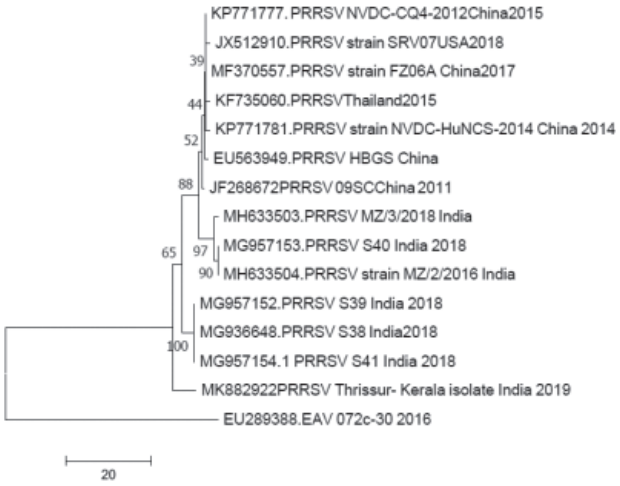

Fig. 7. Neighbour-joining tree constructed from ORF 6 gene sequences showing the phylogenetic relationships between PRRSV isolates. (Horizontal branch lengths are drawn to scale).

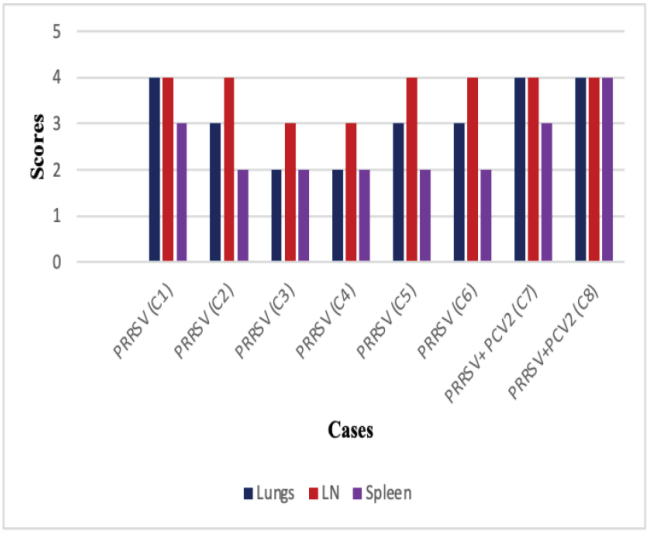

Fig 8. Scores of histopathological lesions of lungs, lymph nodes and spleen. [The Kruskal-Wallis test was used for statistical analysis of histopathological lesions of PRRSV infection in different tissues ($p<0.05$)].

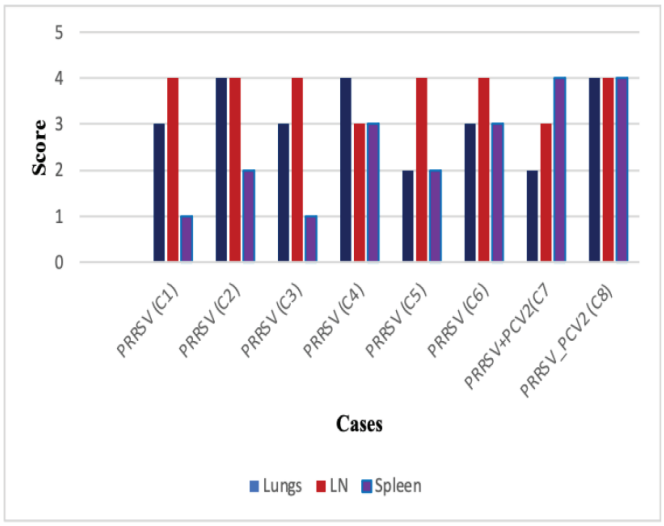

Fig. 9. Scores of viral antigen distribution in lungs, lymph nodes and spleen by immunohistochemistry. [The Kruskal-Wallis test in SPSS (v24.0) was employed for statistical analysis of PRRSV antigen intensity in different tissues ($p<0.05$)].

isolation, IHC, and in situ hybridization in detecting PRRSV infection [25]. Samples from eight swine lungs were positive for PRRSV infection.

Gross lesions in the infection were predominantly evident in the lungs and lymph nodes, characterized by consolidated lungs and moderate to severe lymphadenopathy in all eight swine carcasses. Histopathologically, interstitial pneumonia was considered the hallmark lesion of the PRRSV infection [26]. Lung lesions in PRRSV are qualitatively similar but can vary quantitatively in severity [27]. Lymphoid organs such as tonsils of the soft palate, spleen, and lymph nodes showed moderate to severe lymphoid depletion. Lymphoid depletion was mainly due to the apoptosis caused by the replication of PRRSV [28].

Other gross lesions were splenomegaly, hemopericardium, and hepatomegaly with white foci. These observations were in line with the previous report [29]. On histopathological evaluation, there was moderate to severe inflammatory infiltration by mononuclear cells in the heart, liver, and kidneys [30, 31].

In the present study, IHC was used to detect viral antigens in formalin-fixed paraffin-embedded tissues, which is considered as a specific and sensitive tool in diagnosing PRRSV infection [32]. The most consistent and intense immunohistochemical staining of PRRSV was seen in the lungs, as described in previous works [33]. PRRSV has a high tissue tropism toward macrophages of porcine cell lines [34]. In the lungs, strong positive signals were observed in the cytoplasm of alveolar and interstitial macrophages. Alveolar macrophages are considered the main cellular target for PRRSV replication, followed by pulmonary intravascular macrophages [35]. After initial viremia, PRRSV mainly replicates in the macrophages of the lymphoid system [36]. These IHC findings in lymph nodes and spleen were in concordance with previous reports [37]. IHC results performed in extra lymphoid organs such as the heart, liver, and kidney showed consistent findings in agreement with the earlier report [29]. Lymph nodes had severe and diffusely distributed

lesions in PRRSV-positive cases in all cases [38]. The highest lesion score in lymph nodes might be due to the primary predilection site and extended replication of the virus for a long period [39]. However, statistical analysis of histopathology and IHC scoring showed no significant differences. In the study, only one positive sample was characterized by sequencing of the ORF6 region of the virus. On BLAST analysis of the nucleotide sequence obtained, it was observed that it was similar to the PRRSV sequence (NCBI Accession number MT347586.1) reported from Assam in North-East India. Studies on the characterization of the virus carried out in Kerala have concluded that PRRSV detected in the state was of the Type II North American genotype and that they were genetically diverse. Based on phylogenetic analysis of ORF5 sequences, Kerala isolates were grouped under subgenotype 4 along with other isolates from Kerala, Mizoram, and Assam. Based on Nsp2 gene sequences, they fell under subgenotype 3, with similarities to isolates from Mizoram [40]. Gene sequencing targeting ORF6 of the study identified the isolate as a Type II North American genotype. The comparison of PRRSV isolates in this study revealed a nucleotide identity of 96.45-97.12%, indicating the genetic diversity of PRRSV strains across the world. The isolate in our study exhibited a high nucleotide identity with isolates from China. The phylogenetic tree showed a relationship with other Indian isolates but formed a separate clade. Based on the phylogenetic tree, the present isolate forms a diverse one compared to isolates from other parts of India and neighboring countries. This might be due to the immense mutation rate of the PRRSV among other RNA viruses [41]. Moreover, only a limited number of Indian isolates were available in GenBank. Depositing additional isolates of PRRSV from Kerala would be helpful to improve the understanding of phylogenetic relationships.

PRRSV-positive cases were further investigated to study the coinfection with one or more pathogens, including PCV-2, PPV and CSFV. In this investigation, co-infection of PRRSV with PPV and/or swine fever was not found. Based on the previously reported severity scale, the severity of pneumonia and inflammatory infiltration was of high score and considered qualitatively severe. One of the possible causes of severe lesions is the potentiation of PCV2 replication by PRRSV [42]. Co-infection of PCV2 with PRRSV infection was reported earlier [43], and it has been proven that synergism exists between PCV-2 and PRRSV. Among the factors contributing to this synergy is the immunosuppressive nature of PCV2 [44].

PMWS was one of the probable diagnoses for the two swine with a positive PRRSV coinfection with PCV-2. The PCR detection, the presence of compatible respiratory and digestive clinical signs, characteristic microscopical lesions, and demonstration of PCV2 antigen using IHC within the lesions and will be needed for confirmation of PMWS [45]. Characteristic histopathological lesions of PMWS, such as granulomatous inflammation with the infiltration of epithelioid cells and the presence of amorphous, single or multiple small, intracytoplasmic botryoid inclusion bodies in the cytoplasm of the histiocytes, were demonstrated in these two cases of PCV2 co-infected with PRRSV [46]. Positive PCV2 antigens were also demonstrated in the lungs, lymph nodes and spleen in the two positive cases of co-infections, which were in agreement with the previous reports [9, 47]. A high amount of PCV2 antigens could be detected by IHC in the lymphoid organs, thus confirming the PMWS diagnosis in this study. All of these cases were negative for PPV and CSFV infections. The key to preventing and controlling epidemic diseases is to enhance animal feeding management, biosafety systems, and quarantine, and implement comprehensive preventive and control plans that thoughtfully combine vaccine use [48].

When swine outbreaks of diseases present with respiratory and reproductive symptoms, this study will direct veterinarians to consider PRRSV and PCV2 as crucial differential diagnoses. This study also highlights the need to assess for other co-infections, such as *Mycoplasma hyopneumoniae*, African swine fever, porcine epidemic diarrhea, etc. Future epidemiological surveys should be conducted to understand the prevalence of pathogens and co-infections in different regions of the country and formulate control strategies. Hence, this study will emphasize the necessity of vaccination protocols for controlling these emerging viral diseases in swine herds in India. To comprehend the diversity, distribution, and evolution of the virus, more research on strains that are now circulating in India is important.

CONCLUSION

Based on macroscopic and histological findings, immunohistochemistry, and molecular screening with reverse transcriptase polymerase chain reaction (RT-PCR), eight samples proved positive for PRRSV overall. Immunohistochemistry identified the distribution of PRRSV and PCV2 antigens in tissues, and lesions were of multisystemic types as revealed by pathological examination. Two instances of PCV2-positive cases co-

infected with PRRSV also showed signs of post-weaning multisystemic wasting syndrome (PMWS). The PRRSV strain was determined to have a Type II North American genotype by the investigation. Furthermore, the results validate the reciprocal connection between PRRSV and PCV-2 in PMWS in southern Indian swine herds. Our study offers useful information on PRRSV and highlights the need for continued monitoring of its spread and evolution in India.

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