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Research Article

MOLECULAR CHARACTERISATION AND PHYLOGENETIC ANALYSIS OF PORCINE CIRCOVIRUS-2 ISOLATED FROM INFECTED PIGS IN THE SOUTHERN REGION OF INDIA

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ABSTRACT: Porcine circovirus-associated diseases (PCVAD) caused by Porcine Circo Virus-2 (PCV-2) is an emerging viral disease with serious effects on animal health, food security, and the swine industry. In this study, samples were collected from the PCV-2 suspected swine carcasses presented to the Department of Veterinary Pathology, CVAS, Mannuthy, for necropsy. Out of 39 suspected samples, seven were positive for PCV-2 by polymerase chain reaction targeting the ORF-2 gene, which yielded an amplicon size of 481 bp. The blast analysis sequences of the present isolates showed more than 98 percent homology with other parts of India and foreign isolates. The genotypic analysis revealed different PCV-2 genotypes, *viz.*, PCV-2d (57 percent), PCV-2b (29 percent), and PCV-2h (14 percent), and clusters 13, 11, and 18, respectively, for the first time in Kerala. The microscopic examination revealed lymphoid depletion in the spleen, soft palate tonsils, various lymph nodes, ileal Peyer's patches (IPP), and jejunal Peyer's patches (JPP). There were occasional botryoid inclusion bodies in mucosa-associated lymphoid tissues (MALT) with congestion in various lymph nodes. Based on history and clinical signs, gross, histopathological, and PCR results, and sequence data, the presence of PCV-2-associated systemic disease was confirmed in this study. Altogether, these findings are helpful in further understanding the pathogenesis of PCV-2, which would help to evolve better strategies for improved disease control and prevention in pigs. Future investigations on the pathogenesis of these new genetic variants of isolates obtained in the present study are required for a better understanding of the pathogenesis of the disease.

Keywords: PCV-2, PCR, Phylogenetic analysis, Histopathology.

INTRODUCTION

In recent decades, the swine farming sector in India has made a substantial contribution to the nation's total animal population, which currently stands at 1.7%. Pigs are livestock with a lot of potential because they can give society nutritional security and faster economic returns. These characteristics are the reason why India's swine production grows steadily. Kerala is one of the southern states of India that is prepared to play a significant role in the production of pigs. Despite having 9.06 million pigs, India's pig population is declining by 12% according to the 2019 livestock census. Numerous viral illnesses that impact swine production have the potential to drastically reduce the number of pigs in our state [1]. Swine health is significantly impacted by viral diseases, including foot and mouth disease, classical swine fever, transmissible gastroenteritis, porcine toro virus, porcine respiratory

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and reproductive syndrome (PRRS), infections linked to porcine circovirus type-2, porcine parvovirus infection, infectious epizootic diarrhea, swine flu, etc. Additionally, it has a disastrous economic effect on the worldwide swine business [2]. In the swine husbandry sector, porcine circovirus type 2 (PCV-2) has emerged as a significant disease and financial threat in recent years. The porcine circovirus (PCV) was first discovered in 1974 as a contamination in the PK-15 cell line and was first described the virus as non-pathogenic [3]. Eventually, in 1999, a novel type of illness was discovered in Canada; it was named post-weaning multisystemic wasting syndrome (PMWS) [4].

The PCV-2 is a single-stranded deoxyribonucleic acid (DNA) virus that belongs to the Circoviridae family. It is the primary cause of PMWS, which the American Association of Swine Veterinarians renamed as swine circovirus-associated disease (PCVAD) in 2006. The swine industry, food security, and animal health are all significantly impacted by PCV-2, a new viral infection that has the highest mutation rate and causes a variety of illness symptoms [5]. In cases of PCV-2 infection in Kerala, pathological abnormalities in lymphoid organs, primarily the spleen and lymph nodes, have been well examined [1, 6].

The alterations in mucosa-associated lymphoid tissues (MALT), such as the tonsils of the soft palate, the ileal Peyer's patches, and jejunal Peyer's patches, in PCV-2 infection, are, nevertheless, little described pathologically. In addition to serving as a physical and crucial immunological barrier against infections, the MALT is one of the first places where mucosal immunity is induced [7]. The existing vaccination tactics need to be improved because of the global evolution of many genotypes from field isolates and the high rate of swine mortality [8]. Less research has been done on the genetic diversity of the most common PCV-2 genotypes in Kerala. Understanding the most common genotypes in Kerala is made possible through the sequencing and phylogenetic analysis of PCV-2 isolates.

Therefore, the objectives of the current study were to use polymerase chain reaction (PCR) to detect porcine circovirus-2 (PCV-2) from tissues and to evaluate the gross and histological characteristics of mucosa-associated lymphoid tissues (MALT) and other visceral organs in PCV-2-infected pigs, followed by phylogenetic analysis. Therefore, the current study may further our knowledge of the pathology of PCV-2 on MALT, which may aid in the development of more effective preventative approaches to manage the condition in pigs.

MATERIALS AND METHODS

Sample collection

The samples were collected from 39 suspected swine cadavers, which were brought for post-mortem examination to the Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Keraladuring the period from March 2019 to December 2020. The history revealed symptoms like muscle wasting, pale skin, unthriftiness, respiratory distress icterus, and diarrhea [9]. Pooled tissue samples were collected from the lymph nodes, liver, lungs, kidney, spleen, and tonsils, which were stored in sterile phosphate-buffered saline at -20°C for polymerase chain reaction (PCR).

Polymerase chain reaction

Using the Qiagen DNeasy blood and tissue kit (Catalog number: 69504), total DNA was extracted from tissue samples suspected of having PCV-2 infection.PCV-2 primer sequences (5'CGGATATTGTAGTCCTGGTCG3; 5'ACTGTCAAG GCTACCACAGTCA3') [4] and PCR conditions were used by previous research [6]. The primers obtained in lyophilized form were reconstituted in sterile triple-distilled water to a concentration of 100 Pico moles (pM/µL). This was spun to pellet down the insoluble particles, and the stock solution was distributed into 50µL aliquots and stored at –20°C, at the time of use, they were thawed and further diluted tenfold to obtain a concentration of 10 pM/µL. The PCR was performed in a total volume of 20 µL reaction mixture using 200 µL PCR tubes. PCR tubes containing the mixture were spun briefly and placed in the thermal cycler (Bio-Rad MJ, Mini, Germany). The reaction mixture contained nuclease-free water-6 µL, forward primer-1µL, reverse primer-1 µL, master Mix - 10 µL and DNA sample-2µL. The reaction was carried out with the final volume of 20 µL in the thermal cycler (Eppendorf). Reaction conditions were set at 94°C at 1min for one cycle followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and then the final cycle at 72°C for 10 min. The procedure was repeated for 35 cycles to amplify 481 bp products. On a 2 percent agarose gel, the amplicons produced after 45 minutes of electrophoresis at 70V and 400mAV were analyzed. Using a gel documentation system, the PCR product was recorded in the gel (Bio-Rad Laboratories, USA).

Histopathology of samples

The samples positive for PCV-2 by PCR were subjected to histopathological examination.

Representative samples of lungs, spleen, and lymph nodes were fixed in 10 percent neutral buffered formalin, and processed through the steps of deparaffinization, dehydration, impregnation, and embedding in paraffin. The tissue sections were then cut at 4-5 μm thickness and stained with hematoxylin and eosin [10].

Phylogenetic analysis

Molecular Evolutionary Genetics Analysis (MEGA) software was used to perform phylogenetic analysis on the sequences acquired for this investigation [11]. 65 PCV-2 isolate sequences from different regions of India and other nations were acquired from GenBank (www.ncbi.nlm.nih.gov/genbank/) for phylogenetic analysis. The downloaded sequences were aligned using the MEGA X Clustal W tool, and then they were trimmed to fit the lengths of the sequences found in this investigation. The Jukes-Cantor model and the Maximum Likelihood approach were used to infer the evolutionary history. The evolutionary history of the sequences under analysis was assumed to be represented by the bootstrap consensus tree which was derived from 1000 replicates [12]. The Jukes-Cantor parameter approach [13], the Tamura 3-parameter method [14], and the Kimura 2-parameter method [15] were used to determine evolutionary distances.

RESULTS AND DISCUSSION

Detection of the virus

The samples used for PCR were the pooled organ tissue samples collected from the carcasses that were suspected of having a PCV-2 infection. The DNA extracted from the suspected PCV2 samples was subjected to a purity test. By using spectrophotometry (2000C), it was observed that all the extracted DNA concentration amounts were more than 20 ng/μL, and the OD values were 260/280 of 1.8-2, which signifies that their purity is good. On PCR amplification, the expected amplified products at 481 bp amplicons were observed (Fig. 1). In this study, 39 samples were collected and processed for PCR. It was found that 7 samples out of 39 were positive for PCV-2, and that included 4 suckling and 3 nursery pigs. The disease occurrence rate in our study was 17.94%.

Macroscopic lesions

The carcasses in each case had apparent bony prominences, were emaciated, had rough to dry hair coats, and were blanched (Fig. 2). In most patients, the inguinal lymph node was congested and moderately enlarged (Fig. 3). Severe congestion and edema were seen in the tonsil soft palate (Fig. 4). In the majority of cases, the kidney displayed multi-focal petechial hemorrhages together with icteric changes (Fig. 5).

Microscopic lesions

In the present study, a histopathological study was carried out on the mucosa-associated lymphoid tissue (MALT) and the bone marrow. The tonsils in positive cases revealed mild to moderate lymphoid depletion in follicles and also showed severe congestion in a few cases. Severe lymphoid depletion with viral intracytoplasmic inclusion bodies (botryoid inclusion bodies) and histiocytic infiltration was also observed in the tonsils of all the positive cases (Fig. 6). The ileal and jejunal Peyer's patches exhibited severe lymphoid depletion in almost all the positive samples (Fig. 7 and Fig. 11). One case was seen with washed-out, necrotic Peyer's patches in the ileum. Lymphoid depletion was found to be markedly associated with histiocytic infiltration. Two of the positive samples revealed characteristic amorphous, single, smaller, basophilic intracytoplasmic viral inclusion bodies in the ileum (Fig. 8) and ileal Peyer's patches. Congestion, sloughing of the villi, and ulcers in the intestine were the other notable findings in the intestine.

In most positive cases, severe lymphoid depletion and histiocytic replacement were found in the lymph nodes, and demarcation between the cortex and paracortex was absent in severe cases. Mild to moderate degrees of congestion, lymphoid depletion, and histiocytic infiltration were seen in all other cases. Pulmonary lesions noted in PCV-2-positive cases were severe emphysematous changes with ruptured alveolar walls, mild to moderate degrees of congestion, alveolar septal thickening, broncho-interstitial pneumonia, moderate to severe levels of desquamation of the alveolar epithelium, serous fluid in the alveolar space, and severely altered lung parenchyma. In the heart, hemorrhages in between the myocardial fibers were observed (Fig. 9). The liver showed sinusoidal congestion, central vein thrombosis, and focal infiltration of inflammatory cells in some cases. Disrupted hepatic cords and multifocal hemorrhages were also noticed. The kidney revealed tubular degeneration (vacuolisation) and cloudy swelling in the renal tubular epithelial cells. Varying degrees of congestion, especially the moderate type of congestion, were also seen in the cortex and medulla of the kidneys. In some positive cases, multifocal hemorrhages in between tubules could be noticed (Fig. 10). One

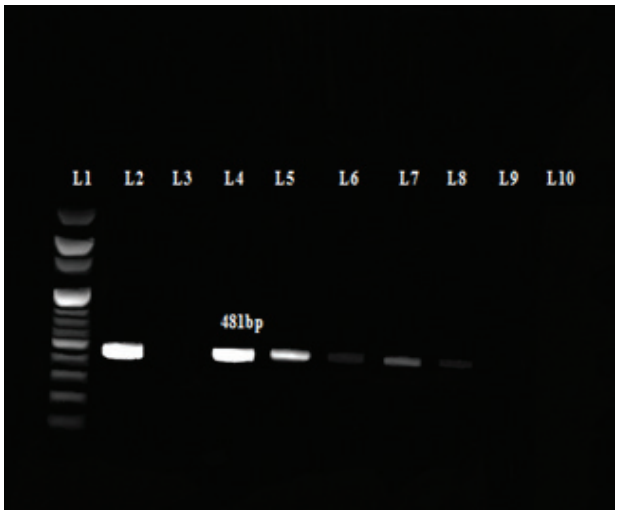


Fig. 1. Agarose gel electrophoresis picture showing 481 bp PCR amplified product of PCV-2. (Lane 1 : DNA ladder, lane 2: positive control, lane 3-negative control, lanes 4 to 8: positive samples; lanes 9 and 10: negative samples).

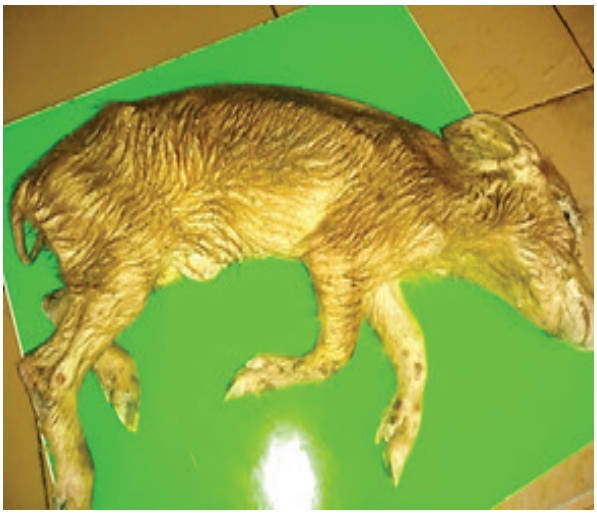


Fig. 2.The blanched, debilitated carcass with rough to dry hair coat, poor body conditions and visible bony prominences.

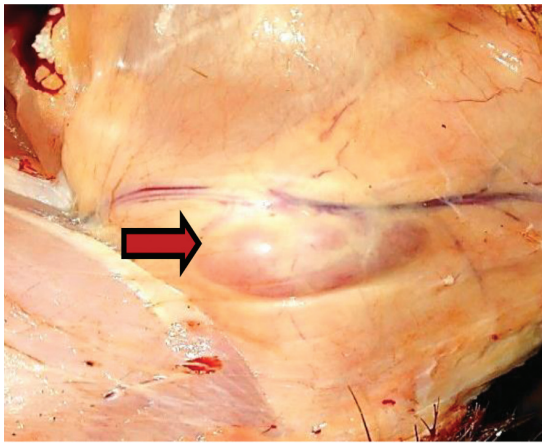


Fig 3.The inguinal lymph node was moderately enlarged (arrow).

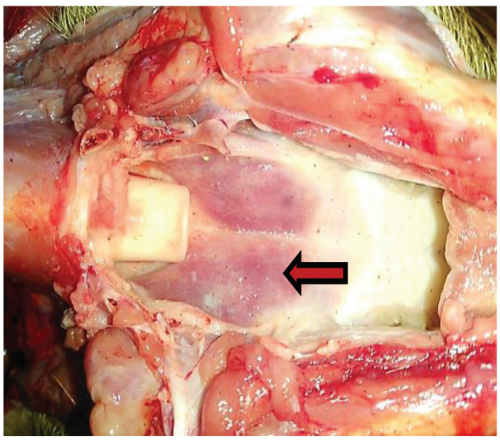


Fig. 4. The soft palate tonsil showed severe congestion and oedema (arrow).

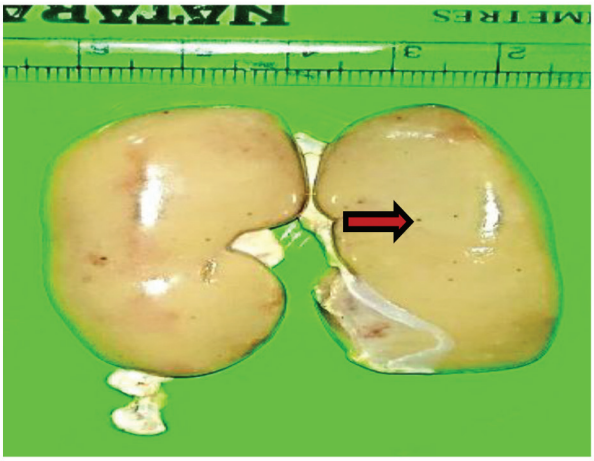


Fig. 5. Kidney showed icteric changes with multi-focal petechial haemorrhages (arrow).

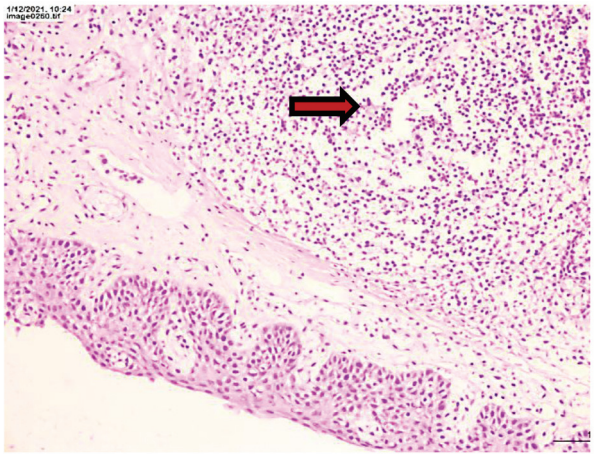


Fig. 6. Tonsilar follicles had severe lymphoid depletion (arrow) (H&E X 200).

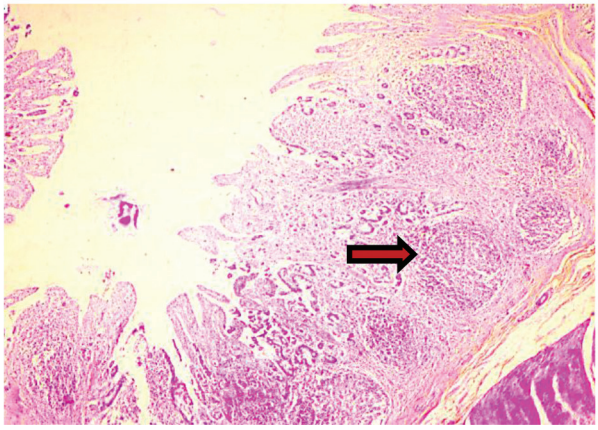


Fig. 7. Peyer's patches in ileum showed severe lymphoid depletion (arrow) (H&E X 200).

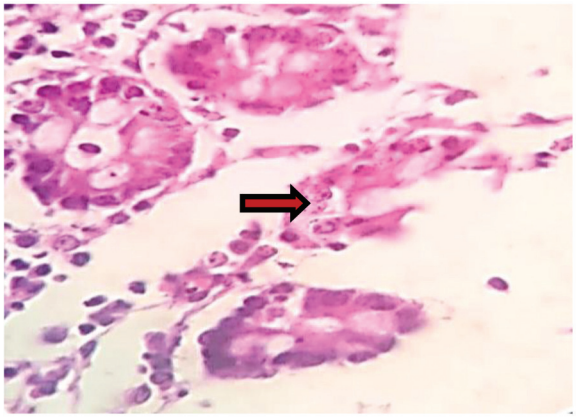


Fig. 8. Botryoid inclusion bodies were seen in the ileum (arrow) (H&E X 1000).

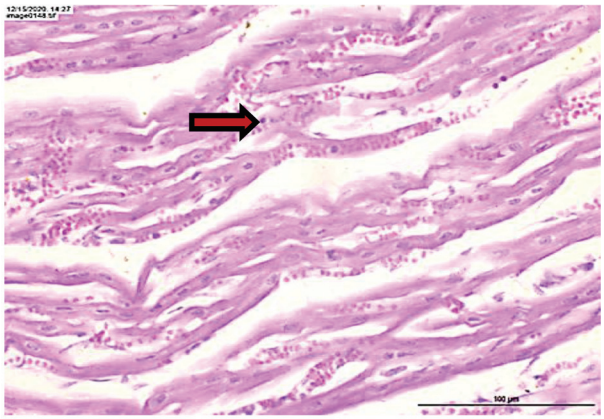


Fig. 9. Heart revealed haemorrhages in between the myocardial fibres (arrow) (H&E X 400).

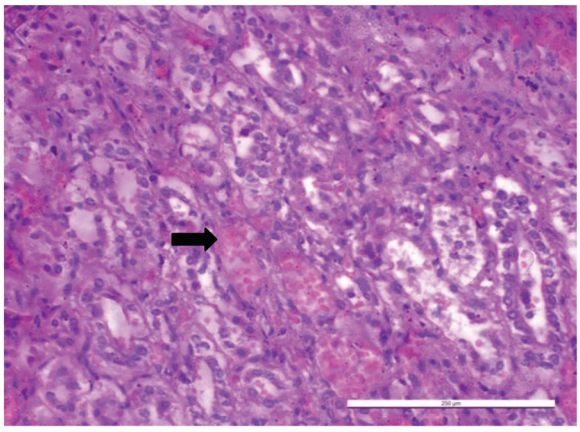


Fig. 10. Kidney showed multifocal haemorrhages (arrow), congestion and degenerative changes in tubules (H&E X 100).

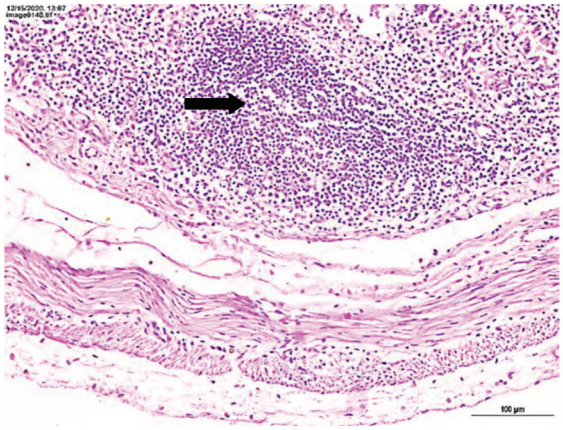


Fig. 11. Jejunal Peyer's patches revealed severe lymphoid depletion (arrow).

case revealed viral inclusion in the tubular epithelium of the kidneys. Moderate to severe lymphoid depletion around the periarteriolar sheaths of the spleen along with moderate histiocytic infiltration were recorded in almost all the cases.

Analysis of PCV-2 gene sequence

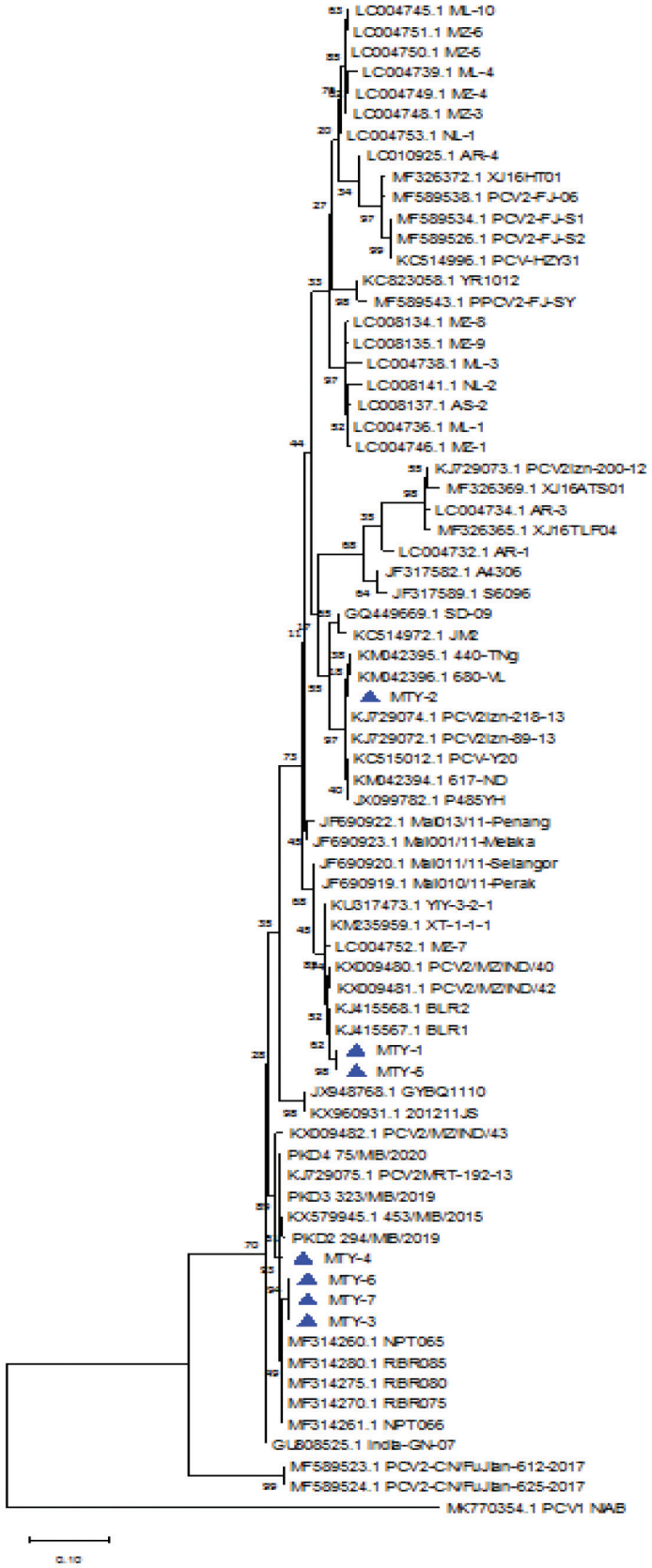
The phylogenetic analysis of the generated sequences was carried out using the MEGA X program in conjunction with the PCV-2 gene sequences from India and other nations that were retrieved from Gen Bank.

Analysis of ORF-2 gene sequences of PCV-2

There were seven PCV-2 positive samples that were processed for sequencing and were later analyzed.

Description	Common Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Porcine circovirus 2 isolate 10v008 cap mRNA, complete cds	Porcine ci...	747	747	100%	0.0	99.51%	702	MK005847.1
Porcine circovirus-2 isolate 3339-PU capsid protein (Cap) gene, compl...	Porcine ci...	747	747	100%	0.0	99.51%	702	KP768484.1
Porcine circovirus-2 isolate 2700-MY capsid protein (Cap) gene, compl...	Porcine ci...	747	747	100%	0.0	99.51%	702	KP768473.1
Porcine circovirus 2 strain CB5923 capsid protein gene, complete cds	Porcine ci...	741	741	100%	0.0	99.27%	702	MT376377.1
Porcine circovirus 2 strain JB5858 capsid protein gene, complete cds	Porcine ci...	741	741	100%	0.0	99.27%	702	MT376309.1
Porcine circovirus 2 strain JB7172 capsid protein gene, complete cds	Porcine ci...	741	741	100%	0.0	99.27%	702	MT376307.1
Porcine circovirus 2 strain CL02, complete genome	Porcine ci...	741	741	100%	0.0	99.27%	1767	MH341483.1
Porcine circovirus 2 isolate 111 capsid protein (ORF2) gene, partial cds	Porcine ci...	741	741	100%	0.0	99.27%	699	MT068287.1
Porcine circovirus 2 isolate 2b_HT34_05_2009_Neumarkt_Upper_Aust...	Porcine ci...	741	741	100%	0.0	99.27%	462	MN150646.1
Porcine circovirus 2 isolate 2b_HT8_02_2010_Langschlag_Lower_Aus...	Porcine ci...	741	741	100%	0.0	99.27%	462	MN150615.1
Porcine circovirus 2 isolate 2b_HT1_01_2002_Gosdorf_Styria capsid p...	Porcine ci...	741	741	100%	0.0	99.27%	462	MN150551.1
Porcine circovirus 2 strain PCV2/Sus_scrofa_domesticus/SLO/JP11/20...	Porcine ci...	741	741	100%	0.0	99.27%	475	MN104844.1
Porcine circovirus 2 strain GX2017-4, complete genome	Porcine ci...	741	741	100%	0.0	99.27%	1767	MK139831.1
Porcine circovirus 2 strain PCV-2|Germany|2018 isolate GER2_3 cap g...	Porcine ci...	741	741	100%	0.0	99.27%	583	MN653193.1
Porcine circovirus 2 isolate GD-1, complete genome	Porcine ci...	741	741	100%	0.0	99.27%	1767	MH920563.1
Porcine circovirus 2 strain HN-ZM-2017, complete genome	Porcine ci...	741	741	100%	0.0	99.27%	1767	MK604515.1
Porcine circovirus 2 isolate WK090315, complete genome	Porcine ci...	741	741	100%	0.0	99.27%	1767	MK347406.1
Porcine circovirus 2 isolate WK070215, complete genome	Porcine ci...	741	741	100%	0.0	99.27%	1766	MK347390.1
Porcine circovirus 2 strain GXNN1603b, complete genome	Porcine ci...	741	741	100%	0.0	99.27%	1767	MH465429.1
Porcine circovirus 2 strain CH/HBJZ4/201407 capsid protein (ORF2) g...	Porcine ci...	741	741	100%	0.0	99.27%	702	KX641118.1
Porcine circovirus 2 strain England/14-P0096-06-14_3 capsid protein g...	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806061.1
Porcine circovirus 2 strain England/21-P465-3-13 capsid protein gene,...	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806051.1
Porcine circovirus 2 strain England/15-P208-7-11 capsid protein gene,...	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806048.1
Porcine circovirus 2 strain England/15-P305-2-12 capsid protein gene,...	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806046.1
Porcine circovirus 2 strain England/15-P24-7-11 capsid protein gene, c...	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806045.1
Porcine circovirus 2 strain England/27-P110-7-12 capsid protein gene,...	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806039.1
Porcine circovirus 2 strain 897624 capsid protein gene, complete cds	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806029.1
Porcine circovirus 2 strain 813310 capsid protein gene, complete cds	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806018.1
Porcine circovirus 2 strain 785698 capsid protein gene, complete cds	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806007.1
Porcine circovirus 2 strain 778290 capsid protein gene, complete cds	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806004.1
Porcine circovirus 2 strain 762454 capsid protein gene, complete cds	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806003.1
Porcine circovirus 2 strain 762450 capsid protein gene, complete cds	Porcine ci...	741	741	100%	0.0	99.27%	702	KY806002.1
Porcine circovirus 2 strain GXNN5, complete genome	Porcine ci...	741	741	100%	0.0	99.27%	1767	KY305202.1
Porcine circovirus 2 isolate F109-4 replicase and capsid protein genes,...	Porcine ci...	741	741	100%	0.0	99.27%	1767	MF142270.1
Porcine circovirus 2 isolate F109-3 replicase and capsid protein genes,...	Porcine ci...	741	741	100%	0.0	99.27%	1767	MF142269.1
Porcine circovirus 2 isolate RBR073 capsid protein gene, complete cds	Porcine ci...	741	741	100%	0.0	99.27%	702	MF314268.1
Porcine circovirus 2 isolate RBR071 capsid protein gene, complete cds	Porcine ci...	741	741	100%	0.0	99.27%	702	MF314266.1

Fig. 12. BLAST analysis of nucleotide sequence of the ORF2 gene of PCV2.



- Sequence from Thrissur districts Kerala, India

Genetic diversity of PCV-2.

No.	Genotype	Cluster
MTY-1	2b	11
MTY-2	2h	18
MTY-3	2d	13
MTY-4	2b	11
MTY-5	2d	13
MTY-6	2d	13
MTY-7	2d	13

Fig. 13. Phylogenetic tree based on the analysis of the 481 bp nucleotide of the ORF-2 gene of PCV-2 isolates from India and other countries.

The 481 bp fragment of the ORF-2 (cap) gene in PCV-2 was taken for analysis. The isolates were serially named as MTY-1, MTY-2, MTY-3, MTY-4, MTY-5, MTY-6 and MTY-7.Among the seven isolates, the isolates MTY-1 (Accession No: MW477471), MTY-5 (Accession No: MW477475), were closely related to the Bhopal isolates KJ415568.1 BLR2, and KJ415567.1 BLR1. More similarities were discovered between isolate MTY-2 (Accession No.: MW477472) and the isolates from Vietnam, KM42396.1 680-VL and KM042395.1 440-TNg. It was discovered that there were similarities between the isolates MTY-3 (Accession No.: MW477473), MTY-4 (Accession No.: MW477474), MTY-6 (Accession No.: MW477476), and MTY-7 (Accession No.: MW477477) and the isolates from Thailand, MF314260.1, MF314260.1, and MF314275.1 (Fig. 12). The PCV-2 isolates were discovered to have a variety of genotypes and clusters, including PCV-2d (4/7), PCV-2b (2/7), and PCV-2 h (1/7), with the corresponding clusters being 11, 13, and 18 (Fig. 13).

As livestock, pigs have a great potential for providing faster economic returns and nutritional security to an economically weaker society. Because of these features, swine production is growing at a steady pace in India. Kerala is one of the southern states of India that has grown up to be a major contributor to swine production. The major hurdle for pig farmers to overcome is infectious diseases, especially diseases of viral origin that have immense power to downturn the pig population of the state. Recently, molecular detection and immune-histochemical diagnosis have confirmed the presence of PCV-2 (PMWS) in Kerala [1, 6]. Lymphoid organs, mainly lymph nodes and spleen, have been extensively studied in PCV-2 infection. However, there is limited pathological data on the changes in MALT, such as soft palate tonsils, ileal, and jejunal Peyer's patches, in PCV-2 infection.

The MALT is one of the initial inductive sites for mucosal immunity and also constitutes a physical and essential immunological barrier against pathogens [7]. Of 39 processed samples, four suckling and three nursery pigs were positive for PCV2 by PCR. Polymerase chain reaction assay is one of the major molecular technique tools that help in the detection of PCV-2 [4]. In the present study, pooled tissue samples (lungs, liver, spleen, kidney, tonsils, and lymph nodes) were used for the isolation of DNA. Out of 39 processed samples, seven were positive for PCV-2. The percentage of positivity was calculated at 17.94 percent. The conventional PCR was done by using nucleocapsid gene-specific primers, which were amplified at 481 bp amplicon fragments [4]. The PCR technique was reported to be more specific and sensitive when compared to IHC, ISH, and virus isolation techniques [16]. Among these positive animals, most of them showed clinical signs such as muscle wasting, unthriftiness, paleness of skin, respiratory distress, visible bony prominences in extremities, chronic ulcers in the skin, peripheral lymphadenopathy, diarrhea, and sometimes icterus [17, 18]. Gross lesions were almost similar in all the cases, which included non-collapsed lungs with varying degrees of congestion, from mild to severe. A few cases revealed consolidation in the cranial lobes of the lungs, and petechial to ecchymotic hemorrhages were observed in a few other cases. Gross lesions, including muscle wasting, lymphadenopathy, non-collapsed lungs, and areas of consolidation mostly in the cranio-ventral lobes of the lung [19, 20]. All cases exhibited a marked bronchial lymph node enlargement. The liver showed a considerable level of enlargement, with congestion ranging from mild to severe, and one carcass revealed a soft, friable liver.In kidneys, gross enlargement and degenerative changes were noticed, as were mild petechial hemorrhages in some cases [16]. In the heart, hydropericardium with severely engorged coronary vessels was also recorded in two cases. Most of the cases revealed systemic lymphadenopathy; almost all the cases have been recorded with severely congested and enlarged mesenteric lymph nodes accompanied by severely engorged mesenteric vessels. Enlargement of inguinal and prescapular lymph nodes was also observed [1, 6]. Spleen mostly appeared with post-mortem changes; some of them showed mild congestion with slight enlargement. A few cases revealed hemorrhagic gastritis and catarrhal enteritis. Microscopically, the predominant lung lesions noticed were severe fibrino-interstitial pneumonia, desquamated bronchiolar epithelium, emphysematous changes with a broken alveolar wall, infiltration of mononuclear cells around the bronchiole, and hemorrhages in the alveolar and bronchiolar areas. These lesions were reported to be consistent with viral infections A study was conducted on 317 cases with pulmonary lesions, and out of these, 88 cases had interstitial pneumonia [21, 22].

Histopathological examination of lymphoid organs such as tonsils, spleen, lymph nodes, ileal, and jejunal Peyer's patches revealed severe lymphoid depletion in the lymphoid follicles, followed by histiocytic

infiltrations in the follicular areas. The absence of demarcation between the cortex and paracortex was a notable finding. Other findings included apoptotic and necrotic changes, especially in the ileal Peyer's patches, with shortened and sloughed-off villi. Lymphoid depletion around the periarteriolar sheath in the spleen was seen in almost all the cases. These findings were previously reported and described by several authors [18, 22, 24]. The characteristic botryoid viral inclusion bodies were also one of the notable findings recorded in the kidney, soft palate tonsils, and ileal Peyer's patches during the microscopical examination. The main reason for the lymphoid depletion was the lysis of virus-infected cells [17]. It indicated the lympho-tropism of the virus, which proved to be the reason for immune suppression in affected animals. The infiltration of histiocytes and monocytes in the lymphoid-depleted area was primarily to remove cell debris and viruses in that particular area [25]. Here, lymphoid depletion and histiocytic infiltration were noticed in all the cases of PCV-2-affected pigs in lymphoid organs, which were in agreement with the previous reports [1, 6]. Here, PCR was used to confirm cases with suspicious gross and histopathological lesions. Since PCV2 is related to various clinical manifestations in pigs, it is associated with various terminologies. Phylogenetic analysis is one of the important genotyping techniques that helps to identify the epidemiology and evolutionary history of viruses. This phylogenetic technique acts as a tool to detect the particular genotype prevalence within certain boundaries of the world, and it helps to identify the possible sources of the introduction of new pathogens in a certain locality or country. In this way, we could identify the various strain outbreaks and accelerate the prevention and control strategies. In our study, the phylogenetic tree was done based on the cap gene ORF-2 [8]. To better understand the evolutionary history of PCV-2 in Kerala, we conducted a detailed study by comparing the isolated sequences with other sequences from India and other countries [11]. For that, a total of 65 sequences were downloaded from GenBank and analyzed with MEGA X software through the Cluster W program. The reliability of the constructed phylogenetic tree was further analyzed by Bootstrap 1000 replications [12]. All seven PCV-2 positive samples, namely, MTY-1, MTY-2, MTY-3, MTY-4, MTY-5, MTY-6, and MTY-7, were sequenced. The phylogenetic tree was constructed based on the maximum likelihood method. It was observed that the isolates MTY-2, MTY-3, MTY-4, MTY-6, and MTY-7 sequence similarity were closely related to Thailand and Vietnam isolates. More than 98 percent of the nucleotide identity was revealed, which confirmed the transboundary migration of the PCV-2. The phylogenetic analysis of PCV-2 showed the relationship of the virus with other Indian isolates, but it formed a separate clade.

The isolates MTY-1 and MTY-5 were closely related to Bhopal isolates from India. The isolates showed three different genotypic variations, namely PCV-2d (57 percent), PCV-2b, and PCV-2h, which formed the three different kinds of clusters 11, 13, and 18. In our study, we found PCV-2d to be the predominant genotype in Kerala. To the best of our knowledge, this is the first study that revealed three different genotypes of PCV-2, namely, PCV-2d, PCV-2b, and PCV-2h, in Kerala. These diversified genotypic variations in PCV-2 could be due to the evolution of the virus through a fast mutation process, which could manipulate the host memory cells and host immune system. Since cross-protection of the genotype vaccination lacks immune stimulation in pigs, this kind of genetic variation in the PCV-2 due to evolution makes the vaccination process very complex, leading to vaccination failure. Similarly, studies revealed the genetic variability in PCV-2 in the Shandong province of China [26]. Even vaccinated animals were seen as susceptible to the newly evolved strain of PCV-2. The vaccines, which were administered through the parenteral route, led to IgG antibody production, which might neutralize the vaccines, and became ineffective. Since our study identified the potential of the virus to affect the MALT, strengthening the MALT system through immuno-modulation techniques could control PCV-2 infections in pigs. Among the variable genotypes, the dominant genotype reported in our study was PCV-2d. It is the most common strain present in Asian countries. PCV-2d has been identified as a dominant genotypic variant in Asiaover PCV-2 genotyping in a previous study conducted in Shandong province in China from 2015 to 2018 [26]. These variations in the genotypes strongly warrant the need for epidemiological surveillance of PCV-2 infections in Kerala. The present work paves the way for a better understanding of the pathogenesis of PCV-2 on MALT, which in turn helps to evolve better prophylactic measures to control disease in pigs. This study revealed the importance of mucosal vaccination by the oro-nasal route. Oro-nasal vaccinations could be employed to provide enhanced protection against the invasive virus, as the parenterally delivered vaccines were neutralized by IgG antibodies. Moreover, these oral

vaccines are very easy to administer to a large population of animals. The main limitation of the current study was the smaller sample size. Further investigations for new genetic variants of isolates obtained in the present study are required to understand the diversity in pathogenesis. Furthermore, the present study warrants the emergence of novel genetic variants in the field condition as well as the need for genotype-specific vaccines. However, further studies are still needed to better understand this "known unknown" PCV-2 and its expanding strains and future challenges.

CONCLUSION

We have conducted a detailed study on molecular characterization of the PCV-2 virus identified in the pig population of Kerala and also analyzed the phylogenetic relationship between the PCV-2 isolates from Kerala with other foreign PCV2 isolates. The epidemiology of the PCV-2 isolates from Kerala was similar to the isolates of Bhopal, Thailand, and Vietnam isolates. Accordingly, our work opens the door to a deeper comprehension of the epidemiology of PCV2 viruses that are prevalent in Kerala and may also aid in the prevention of PCV-2 infection.

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REFERENCES

1. Sairam R, Krishna BD, Krithiga K, Sajitha IS, Priya PM *et al.* Molecular and pathological studies of post-weaning multi-systemic wasting syndrome among piglets in Kerala, India. Explor Anim Med Res. 2019; 9(2), 137-144.

2. Kedkovid R, sirisereewan C, Thanawongnuwech R. Major swine viral diseases: an Asian perspective after the African swine fever introduction. Porc Health Manag. 2020; 6: 1-11.

3. Tischer I, Mields W, Wolff D, Vagt M, Greim W. Studies on epidemiology and pathogenicity of porcine circovirus. Arch Virol. 1986; 91: 271-276.

4. Ellis J, Krakowka S, Lairmore M, Haines D, Bratanich A *et al*. Reproduction of lesions of postweaning multisystemic wasting syndrome in gnotobiotic piglets. J Vet Diagn Invest. 1999; 11: 3-14.

5. Correa-fiz F, Franzo G, llorens A, Huerta E, Sibila M. Porcine circovirus 2 (PCV-2) population study in experimentally infected pigs developing pcv2-systemic disease or a subclinical infection. Sci Rep. 2020; 10: 1-9.

6. Keerthana J, Abraham MJ, Krithiga K, Priya PM *et al.* Pathological and immunopathological studies on naturally infected cases of porcine circovirus 2 in Kerala. Int J Livest Res. 2017; 12: 81-86.

7. Shi F, Li Q, Zou Z, Wang Y, Hou X *et al.* The changes of immune-related molecules within the ileal mucosa of piglets infected with porcine circovirus type 2. J Vet Sci. 2020; 21:42-46.

8. Tsai GT, Lin YC, Lin WH, Lin JH, Chiou MT *et al.* Phylogeographic and genetic characterization of porcine circovirus type 2 in Taiwan from 2001-2017. Sci Rep. 2019; 9: 1-13.

9. Joseph J, Balakrishnan-Nair DK, Anoopraj R, Priya PM, Ravishankar C *et al.* Histopathological and immuno-histochemical evaluation of male and female reproductive systems of porcine circovirus-2 infected pigs. Explor Anim Med Res. 2023; 13(2), DOI: 10.52635/eamr/13.2.169-177.

10. Suvarna K, Layton C, Bancroft J. Bancroft's theory and practice of histological techniques. (8th edn.). Elsevier health sciences, 2019; 672.

11. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. Mega X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018; 35: 1547-1549.

12. Felsenstein J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution. 1985; 39: 783-791.

13. Jukes TH, Cantor CR. Evolution of protein molecules. In: Munro HN. (Editor.), Mammalian Protein Metabolism. Academic Press, New York, 1969; 121-132.

14. Tamura K. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C content biases. Mol Biol Evol. 1992; 9: 678-687.

15. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol. 1980; 16: 111-120.

16. Kim J, Chae C. A comparison of virus isolation, polymerase chain reaction, immunohistochemistry and in situ hybridization for the detection of porcine circovirus 2 and porcine parvovirus in experimentally and naturally coinfected pigs. J Vet Diagn Invest. 2004; 16: 45-50.

17. Segales J, Domingo M. Postweaning multisystemic wasting syndrome (PMWS) in pigs: A review. Vet Q. 2002; 24: 109-124.

18. Rosell C, Segales J, Plana-d J, Bala M, Roiez-arrioja GM *et al.* Pathological, immunohistochemical and in-situ hybridization studies of natural cases of postweaning multisystemic wasting syndrome (PMWS) in pigs. J Comp Pathol. 1999; 120: 59-78.

19. Parthiban S, Ramesh A, Karuppannan AK, Dhinakar RG, Hemalatha S *et al.* Molecular prevalence of porcine

circovirus 2 infection: foremost report in Southern states of India. Explor Anim Med Res. 2022; 12(1), DOI: 10.52635/ eamr/ 12.1.99-108.

20. Anoopraj R. Studies on the pathology of respiratory diseases in pigs caused by porcinecircovirus type 2. Ph.D. thesis, Indian Veterinary Research Institute, Izatnagar, India. 2014; 325.

21. Aramouni M, kekarainen T, Ganges l, Tarradas J, Segales J. Increased viral load and prevalence of torque teno sus virus 2 (ttsuv2) in pigs experimentally infected with classical swine fever virus (CSFV). Virus Res. 2013; 172: 81-84.

22. Tico G, Segales J, Martinez J. The blurred border between porcine circovirus type 2- systemic disease and porcine respiratory disease complex. Vet Microbiol. 2013; 163: 242-247.

23. Allan GM, Ellis JA. Porcine circoviruses: a review. J Vet Diagn Invest. 2000; 12: 3-14.

24. Wilfred E, Mutebi F, Mwiine, FN, James OA, Lonzy O. Porcine circovirus type 2-systemic disease on pig farms and associated knowledge of key players in the pig industry in central Uganda. Int J Vet Sci Med. 2018; 6: 178-185.

25. Ghebremariam MK, Gruys E. Postweaning multisystemic wasting syndrome (PMWS) in pigs with particular emphasis on the causative agent, the mode of transmission, the diagnostic tools and the control measures-a review. 2005; Vet Q. 27: 105-116.

26. Ma Z, Liu M, Liu Z, Meng F, Wang H *et al.* Epidemiological investigation of porcine circovirus type 2 and its coinfection rate in Shandong province in China from 2015 to 2018. BMC Vet Res. 2021; 1: 1-9.

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