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RESEARCH ARTICLE

# Pathological and molecular diagnosis of porcine Circovirus 2 in slaughtered pigs of India

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## ABSTRACT

Porcine Circovirus 2 (PCV2), an emerging viral pathogen of pigs is distributed worldwide causing huge economic loss to the swine industry. Despite its high prevalence globally, very little or no reports are available regarding the prevalence of porcine circovirus-2 (PCV-2) in slaughtered pigs. The objective of our study was to estimate the prevalence of PCV2 in Indian slaughtered pigs using pathological and molecular techniques. Out of 968 morbid tissue samples (lungs-484; lymph nodes-484) collected from slaughtered pigs showed the high prevalence of PCV2 (65.28%) by PCR targeting ORF2 gene showing the amplification of 481 bp. Among 632 positive morbid tissues, lungs (52.89%) showed higher detection of PCV2 DNA as compared to lymph nodes (12.39%). Microscopically, affected lungs showed the predominant lesions of subacute interstitial pneumonia, proliferative and necrotizing pneumonia with suppurative bronchopneumonia. The lymph nodes showed the predominant lesions of lymphoid depletion with infiltration of histiocytes and macrophages in the medullary cords and sinuses. PCV2 antigen was immunohistochemically demonstrated in the alveolar macrophages and mononuclear cells in the lymph nodes confirming the association of PCV2 with associated pathologies. The phylogenetic analysis revealed that the PCV2 isolates circulating in pigs belonged to PCV2d-2 genotype. In conclusion, this study shows that PCV2d-2 is an emerging genotype circulating in Indian slaughtered pigs, which warrants the immediate implementation of PCV2 vaccination to aid the development of prevention and control strategies.

**Keywords:** Histopathology, immunohistochemistry, molecular diagnosis, PCV-2, PCV2d-2 genotype, phylogenetic analysis

## INTRODUCTION

Porcine circovirus (PCV), a single-stranded, circular DNA virus belonging to the Circovirus genus of the Circoviridae is an emerging ubiquitous viral pathogen confronting swine industry worldwide[1,2]. It is responsible for porcine circovirus virus-associated disease (PCVAD), which includes a number of diseases such as post-weaning multi-systemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), granulomatous enteritis, porcine respiratory disease complex, reproductive failure and acute pulmonary edema based on the virus, hostimmunity, co-infections and other environmental characteristics[3,4]. Its high prevalence has been reported in different swine rearing countries of the globe such as North and South America, Europe, Asia, Oceania, Middle East, and the Caribbean including India[1,4,5]. Currently, PCVs are classified into four genotypes such as PCV1, PCV2, PCV3 and identified recently PCV4; out of which PCV2 is frequently reported in global swine industry[1,6,7]. Recently, PCV2 is divided into eight different genotypes: PCV2a, PCV2b, PCV2c, PCV2d, PCV2e including recently reported PCV2f, PCV2g, and PCV2h genotypes based on ORF2 classification criteria[8]. Recent reports showed the continuous evolution of PCV2 leading to emergence of novel strains due to genetic shift[1,9]. The first identified PCV2a, the oldest genotype is used in available commercial PCV2 vaccines due to its predominance till 2003. Later on, global genotype shift was observed from PCV2a to PCV2b and recently to PCV2d in global swine production[1,9]. PCV2d is further classified to PCV2d-1 and PCV2d-2. Out of these 2 subtypes, PCV2d-2 is frequently reported worldwide with increased virulence[1,9]. The frequent reports of high prevalence of various genotypes of PCV2 along with intergenotypic recombination in different geographical regions of India suggest that PCV2 is likely to be endemic in India[10-15]. Moreover, PCV3 has been reported in a pig farm of Chhattisgarh with the history of severe reproductive failure, piglet mortality and dermatitis[5]. Out of its various genotypes, PCV2d genotype is frequently reported

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in Indian pig population[7,12,14,15]. However, majority of the cases were reported from the organized farms only. The use of histopathology and immunohistochemical (IHC) techniques in association with molecular diagnosis has been an important source of information for sanitary monitoring of pigs for the identification and monitoring the circulation of infectious pathogens[16]. Diagnosis of PCVAD is primarily based on clinical signs, histologic lesions, and detection of PCV-2 antigens or DNA within characteristic lesions. Despite continuous reports of newly emerging strains and global genotype shifts, there is little information available regarding the prevalence of PCV2 in slaughtered pigs of India. The objectives of this study were to broaden our knowledge of prevalence of PCV2 with associated pathologies in slaughtered pigs using pathological and molecular techniques.

## MATERIALS AND METHODS

A total of 968 morbid tissue samples (lungs-484; mesenteric lymph nodes-484) from 484 sacrificed pigs (cross bred-302, desi-182) of aged < 6 months age (121) and adults (363) were collected out of 982 pig carcasses examined in the slaughterhouses at various slaughterhouses belonging to Chandigarh (Punjab, 78), Bareilly (Uttar Pradesh, 148), and Mumbai (Maharashtra, 258) using random sampling method. These slaughterhouses were targeted because of high the turnover of pigs from densely pig-rearing areas. After the evisceration, thin (< 5mm) tissue pieces from lungs and lymph nodes were aseptically removed and examined for gross pathological lesions. All the lungs (484) showed varying degrees of pulmonary lesions, while 29 lymph nodes were enlarged. The tissues were fixed in 10% neutral buffered formalin (NBF) for histopathology and immunohistochemistry (IHC) studies. The aseptically collected tissues were transferred to the laboratory in a sterile container on ice and stored at -20$^{O}$C for the molecular detection of PCV2.

### Histopathological investigation

The 10% NBF fixed tissues were routinely processed followed by staining with routine Hematoxylin and Eosin (H&E) method[17]. The stained slides were examined under the microscope, and the alterations, if any were photographed (Olympus BX41, USA). The duplicate paraffin tissue sections of lungs and lymph nodes were taken on poly-L-lysin coated glass slides for the *in-situ* demonstration of PCV-2 antigen in lungs and lymph nodes.

### Immunohistochemistry investigation

Briefly, the tissue sections were deparaffinized, rehydrated, and treated with proteinase-k solution (Abcam, USA) for 15 min to unmask the antigen, followed by washing with phosphate buffer saline - tween (PBST) for 2X of 5 min each. The quenching was done by adding 0.5% $H_2O_2$ for 30 min to block the endogenous peroxidase activity. The prediluted horse serum (Vectorlab, USA) was applied for 30 min at room temperature (RT) in the humid chamber to stop nonspecific binding. Then, the sections were incubated with PCV-2 capsid rabbit polyclonal antibody (1:200) for overnight at 4$^{O}$C in humidity chamber followed by incubating the sections with prediluted ImmPRESS® HRP universal secondary antibody (MP-7500, Vector laboratories, USA) for 30 min. Afterwards, the sections were added with ImmPACT® DAB Substrate, (HRP) (SK-4105, Vector laboratories, USA) for 30 sec till development of the color. After the color development, sections were counter stained with Mayer's haematoxylin, and mounted on the aqueous mountant (C9368, CC/Mount, Sigma-Aldrich, USA) for visualization of signals under the microscope. Negative controls were run for each block tested with substitution of similar dilutions of PBS with 1% BSA for primary antibody and mouse isotype control antibodies (ThermoFischer Scientific, USA) as secondary antibody to rule out the false positive staining.

### Molecular investigation

The DNA was extracted from the lungs and mesenteric lymph nodes using commercially available DNeasy Blood and Tissue Mini Kit (Qiagen, USA) as per manufacture's protocol. The viral genomic DNA was further subjected to PCR using the previously reported primer sets targeting the ORF2 region of PCV2[18].

### Sequencing and phylogenetic analysis

The amplified 4 PCR amplicons were purified by using Gene JET PCR purification kit (Thermo

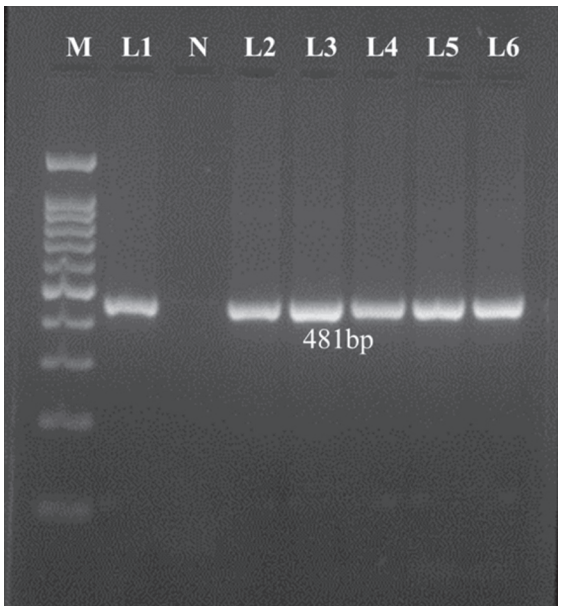


**Fig. 1.** Molecular detection of PCV2 showing amplified product of 481 bp for ORF2 gene, Lane M: Ladder (100 bp), L1: Positive control, L2: Negative control, L3: Lungs, L4: Lungs, L5: Lymph node, L6: Lymph node positive for PCV2.

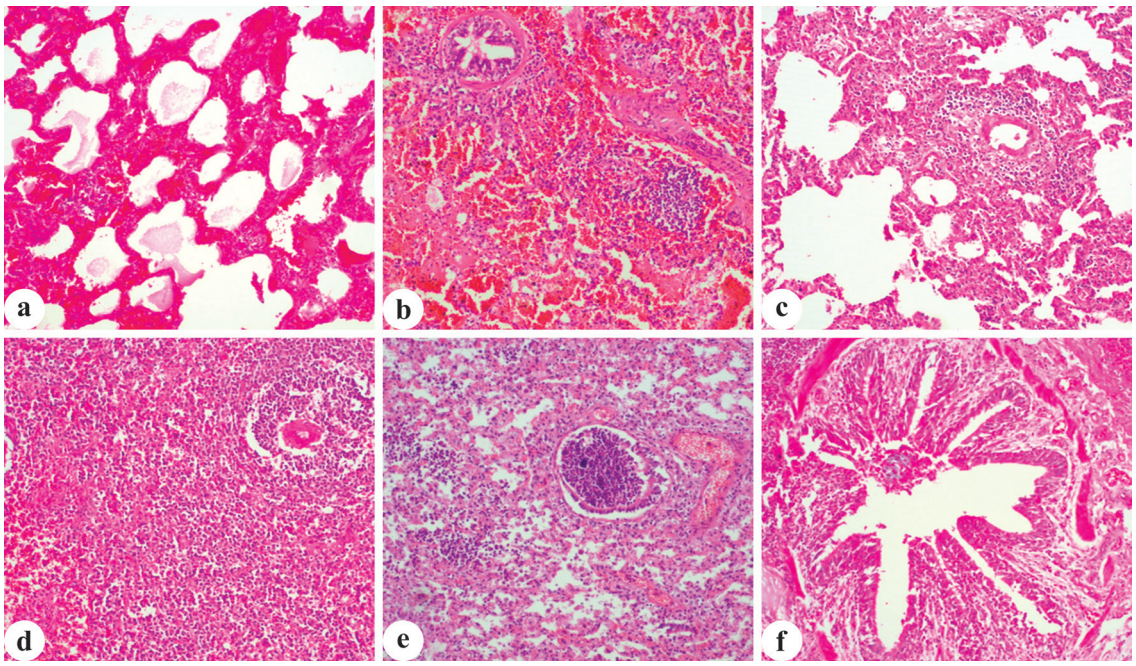


**Fig. 2.** Histopathological lesions in pigs of naturally infected with PCV2 showing **a.** Interstitial pneumonia with oedema (H&E X100); **b.** Extensive areas of haemorrhages in the pulmonary interstitium (H&E X100); **c.** Interstitial pneumonia with perivascular lymphocytic infiltrate (H&E X100); **d.** Proliferative and necrotizing pneumonia (H&E X100); **e.** Suppurative bronchopneumonia (H&E X100); **f.** Bronchiolitis fibrosa obliterans (H&E X200).

Fisher Scientific, USA) and then these got sequenced commercially (Eurofins, Bangalore). The sequence data (FASTA files) were analyzed using 'EditSeq' programme of 'Lasergene' version 10 (DNASTAR Inc, USA). The nucleotide sequences of the isolates belonging to different genotypes of PCV were retrieved from GenBank and aligned with nucleotide sequences of the study isolates by using the ClustalW method. The evolutionary history was

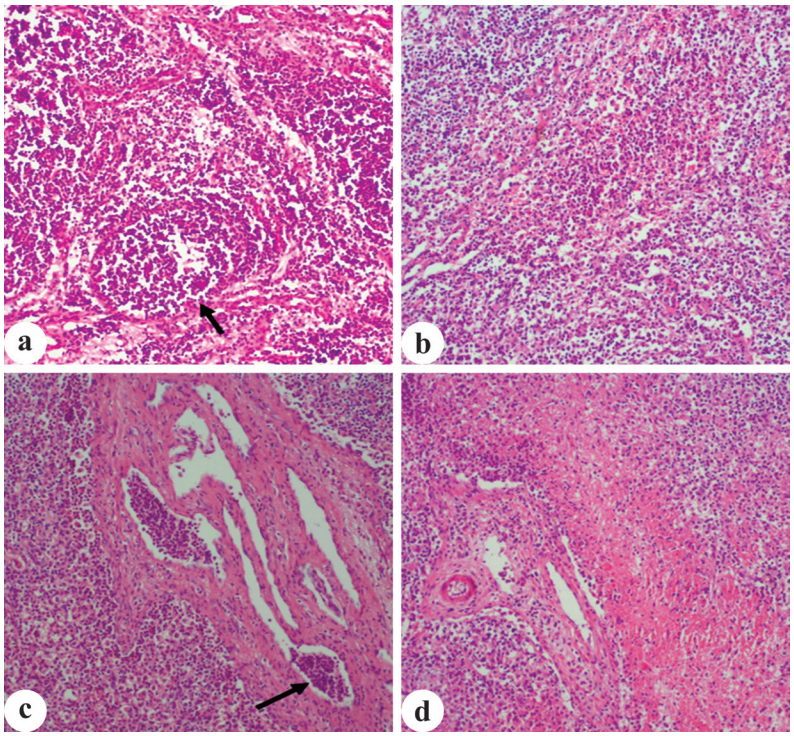


**Fig. 3.** Histopathological lesions in lymph nodes of naturally infected with PCV2 showing **a.** Lymphoid depletion (arrow) (H&E X100); **b.** Marked infiltration of macrophages and histiocytes in medullary sinus (H&E X100); **c.** Purulent lymphadenitis showing infiltration of neutrophilic exudates (arrow) within lumen of blood vesssel (H&E X100); **d.** Large area of necrosis in the medulla (H&E X100).

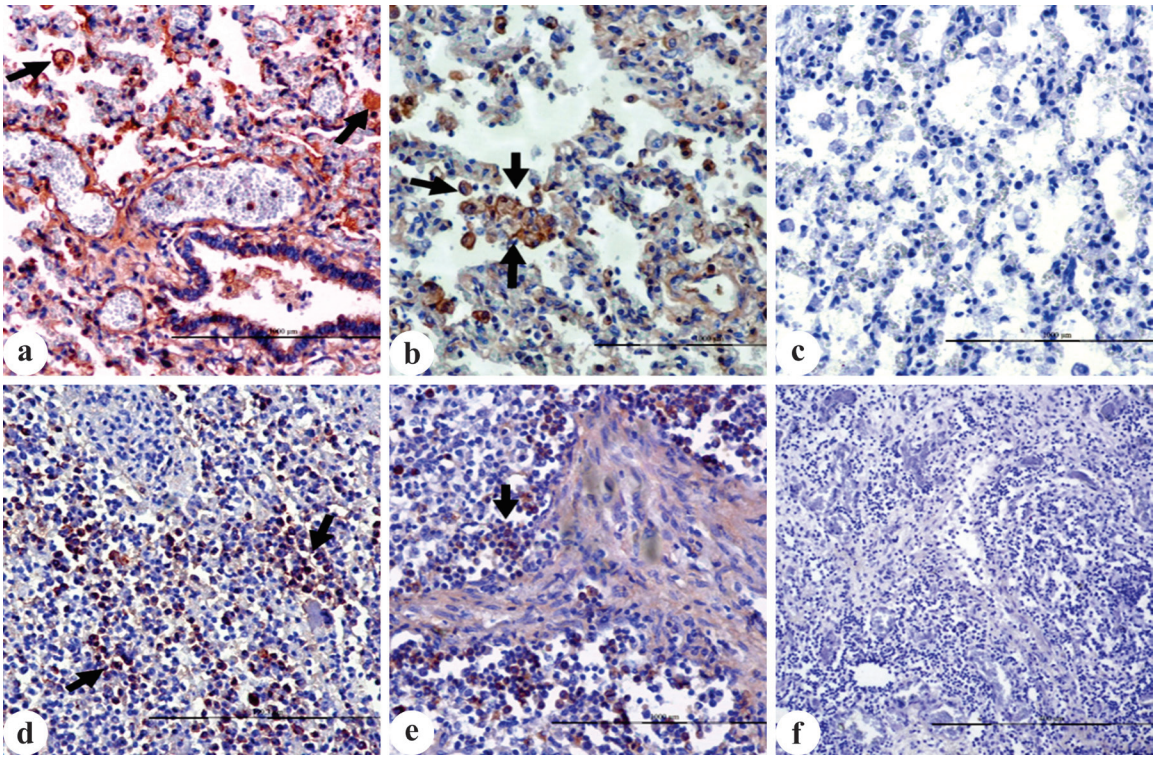


**Fig. 4.** The immunolocalization of PCV2 antigen in lungs and lymph nodes of naturally infected slaughtered pigs **a.** Alveolar macrophages (arrow), vessel wall and inflammatory cells in the vascular lumen, lungs; **b.** Alveolar macrophages, lungs; **c.** Absence of immunoreactivity, negative control, lungs; **d,e.** Mononuclear cells and histiocytes (arrow), lymph nodes; **e.** Negative control, lymph node (IHC X40 bar 1000 μm).

inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.74952771. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 4). The analysis involved 17 nucleotide sequences with codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 752 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

## RESULTS

Out of 968 morbid tissues tested, 632 tissues from 354 pig carcasses showed the amplification of ORF2 gene with product size of 481 bp specific to PCV-2 (65.28%, 632/968) (Fig. 1). Out of 632 positive cases, lungs (52.89%) showed higher detection of PCV2 than lymph nodes (12.39%). Among the different states, Uttar Pradesh showed higher positivity (41.9%), while Chandigarh showed lowest (11.6%) detection of PCV2. The high prevalence was observed in adult age group (78.4%) than young age group (21.5%). The crossbred pigs (61.9%) showed higher detection of PCV2 as compared to desi breed.

Grossly, the PCV2-infected lungs were heavy, non-collapsible, diffusely mottled red to purple, and rubbery in majority of the cases (87.85%) with interlobular edema (60.74%). The mediastinal lymph nodes were enlarged and oedematous. The lungs showed the lesions of interstitial pneumonia (Fig. 2a) accompanied with diffuse congestion, hemorrhages in the interstitium (Fig. 2b), vasculitis (Fig. 2c) accompanied with infiltration of lymphocytes, macrophages in the alveolar lumen, alveolar septae and at places in the connective tissue of the bronchi/bronchioles. The bronchial and bronchiolar epithelium of the affected lungs showed hyperplasia, desquamation, and desquamated epithelium admixed with inflammatory cellular exudates within the lumen. The interalveolar septa was thickened with infiltration of mononuclear cells, and macrophages. The pleura was moderately thickened due to infiltrations of fibrino-cellular exudates. The predominant histologic diagnosis was interstitial pneumonia associated with proliferative and necrotizing pneumonia (58.24%) (Fig. 2d), suppurative bronchopneumonia (41.69%) (Fig. 2e), fibrinonecrotizing bronchopneumonia (21.85%),

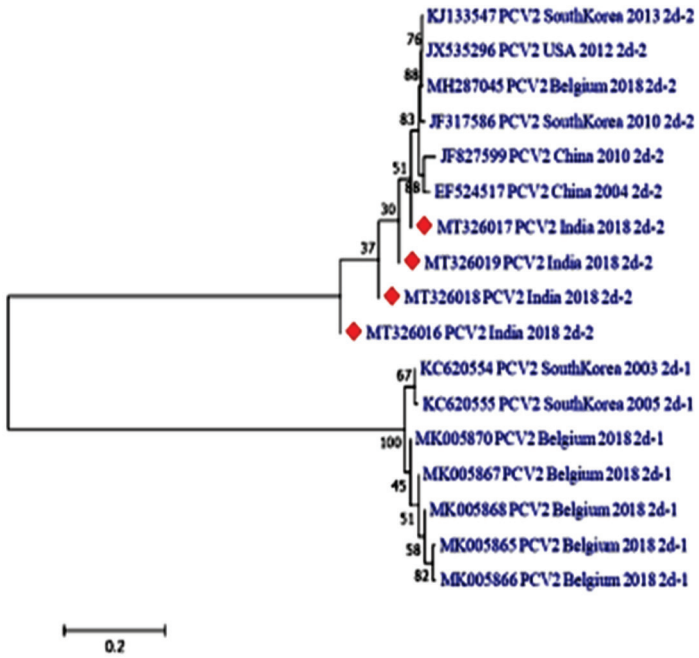


**Fig. 5.** Phylogenetic tree of the ORF2 nucleotide sequences of various genotypes of PCV2 using the Neighbor-Joining method in MEGA 7 showing the presence of PCV2d-2 genotype. Samples with solid Red coloured triangles represent Indian PCV2 isolates included in the present study.

peribronchiolar fibrosis and bronchiolitis fibrosa obliterans (Fig. 2f) were observed. In 49 cases, lymphoid depletion with histiocytic infiltration was observed in bronchus-associated lymphoid tissue (BALT). The majority of the lymph nodes did not show any appreciable gross lesions except multifocal areas of necrosis was observed in 13 lymph nodes (10.83%). Microscopically, majority of the lymph nodes (89.26%) showed variable degree of lymphocyte depletion (Fig. 3a) with loss of follicular architecture with infiltration of histiocytes in the medullary cords and sinuses (Fig. 3b). Majority of the lymph nodes showed mild to moderate degree of lymphoid depletion. A total of 11 lymph nodes showed indistinct lymphoid follicles in one or more lymph nodes. The purulent lymphadenitis (Fig. 3c) was also observed in 21 cases. Nine lymph nodes showed coagulation necrosis affecting the wide areas of lymph node parenchyma (Fig. 3d). Besides, various lesions like haemosiderosis, oedema, fibrosis and multinucleated giant cells in the lymphoid follicles were detected in few cases (5.73%).

Immunohistochemically, abundant immunosignaling of PCV2 antigens were detected consistently intracytoplasmically in the inflammatory exudates especially alveolar macrophages, lymphocytes (Figs. 4a, b) and rarely intranuclearly in epithelial cells. The immunoreactivity was also noticed in vessel wall with few inflammatory cells present in the vascular lumen (Fig. 4a). The alveolar, bronchial and bronchiolar epithelial cells also showed intracytoplasmic immunoreactivity for PCV2 antigen. The presence of PCV2 antigen was also detected in bronchial and vascular smooth muscle cells and in epithelial cells of bronchial submucosal glands. In lymph nodes, abundant immunosignaling for viral antigen was noticed in the lymphocytes, histiocytic and dendritic cells of the medullary region (Figs. 4d, e). The negative controls of lungs and spleen did not show any immunoreactivity for PCV2 antigen (Figs. 4c, f).

The comparative phylogenetic analysis of 4 PCV-2 positive Indian isolates (MT326016, MT326017, MT326018 and MT326019) with the sequences of PCV-2 in NCBI database showed 98% similarity with PCV2d sequences. Further, our sequences with the sequences of 2 different genotypes (PCV2d-1 and PCV2d-2) of PCV2d sequences downloaded from the database formed clad with PCV2d-2 genotype sequences while forming separate clad from PCV2d-1 sequences (Fig. 5). The sequences of Indian isolates belonging to PCV2d-2 genotype were closely related to the sequences of the isolates reported from China, South Korea, Belgium and USA isolates.

## DISCUSSION

Porcine circovirus type 2 (PCV2), recognized as emerging global viral pathogen is frequently reported from slaughtered pigs worldwide[2,6,19]. Despite its high economic importance, the paucity of literatures regarding the prevalence of PCV2 with the associated pathologies in Indian slaughtered pigs prompted us to take the present investigation.

Slaughterhouse surveys are essential components of

infectious disease control and eradication programmes worldwide being the source being the source of providing helpful information on the prevalence of economically important animal diseases including diseases of zoonotic importance[20]. The detection of PCV2 by PCR is considered as a rapid tool in identifying PCV2 infections in pigs[21]. Therefore, the present study used PCR as PCV2 detection method. The overall prevalence of PCV2 in the slaughtered pigs was 65.28%. In our case, the possible reason for the higher prevalence in Uttar Pradesh might be due to slaughtering of pigs from different states of North as well as North-Eastern India. Uttar Pradesh has the second highest pig population (1.35 million) in India (19th Livestock Census, 2012), thus higher numbers of pigs being slaughtered. The high prevalence of PCV2 in slaughtered pigs was reported in earlier reports[2,6,19,22]. The complex interaction of multi-etiological factors such as infectious pathogens, environmental factors, and management and husbandry practices play pivotal role in the development of clinical disease. The high evidence of the disease in the older pigs (above 6 months of age) in comparison to the younger pigs (< 6 months of age) might be due to increased exposure of pigs (mainly horizontal transmission) to infection with the advancement of age. The higher detection of PCV2 in crossbred pigs as compared to desi breed might be due to more number of pigs received to the slaughterhouse for slaughtering. The higher prevalence of PCV2 was detected in lungs than lymph nodes suggest the major contribution of PCV2 in pneumonic lesions. PCV2 is the major viral pathogen associated with porcine respiratory disease complex in the slaughtered pigs[1,23-25]. The affected lungs showing the histopathological lesions of interstitial pneumonia, proliferative and necrotizing pneumonia with suppurative bronchopneumonia are in agreement with the previous reports[9,12,15,26,27]. The predominant lesions of lymphoid depletion with infiltration of macrophages and histiocytes in the lymph nodes were similar to previous observations in weaned pigs diagnosed with PMWS[11,28,29]. The lymphoid depletion might suggest the PCV2 associated immunosuppression which predisposes the animal to secondary bacterial infection[12,30].

The IHC study is a reliable method for confirmation of role of PCV-2 in swine diseases. The abundant immunoreactivity of PCV2 antigens in lungs and lymph nodes at the lesional sites confirm the role of PCV2 with the associated pathologies. The detection of PCV2 antigens in the bronchial, bronchiolar, alveolar epithelial cells and vascular smooth muscle cells in the present study was similar with the earlier observation[26,31]. The detection of PCV2 antigens in the alveolar macrophages suggest the replication of PCV2 in macrophages[31]. The immunodetection of abundant PCV2 antigens in the lymphocytes and histiocytes suggest the uptake of viral antigens in the antigen-presenting cells, which later on might be transported either intracellularly or free in lymph and/or blood. The predominant lesions of histiocytic infiltration in the lymph nodes in our case corroborated with the previous reports showing PCV2 associated lymphadenopathy[31]. The immunoreactivity of large amounts of PCV2 antigen in the wall of the blood vessels of lungs and lymph nodes suggests that the vascular system may be important in the pathogenesis of PCV2[32].

In the phylogenetic analysis, it was found that Indian PCV2 isolates matched 98% with PCV2d isolates from the database which suggest the prevalence of PCV2d genotype in Indian slaughtered pigs. The higher detection of PCV2d genotype in Indian pig population is similar with the previous findings[7,12,33]. Further, all 4 isolates forming clade (red, highlighted with red solid triangle marker) with PCV2d-2 genotype suggest that the PCV2d isolates belongs to PCV2d-2 genotype. The circulation of PCV2d-2 genotype in Indian pig population has been reported in previous reports[13,34]. The high prevalence of PCV2d-2 genotype suggests that the genotype PCV2d-2 is getting endemic within the slaughtered pigs of India.

## CONCLUSION

From the present investigation, it may be concluded that PCV2d-2 genotype is prevalent in Indian slaughtered pigs based on pathological and molecular findings. This findings suggest the need for immediate use of PCV2 vaccine in pigs along with designing effective preventive and control strategies to minimise the economic loss to the farmers. However, investigation on large number of samples with continuous surveillance covering the slaughterhouses from various geographical regions of India will give the detailed insight into identification of newly emerging strains and genotype shifts in swine population which will pave the way for the effective control of the disease.

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