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Studies on syndemic infection of porcine reproductive and respiratory syndrome virus with porcine circovirus 2 in backyard pigs of Mizoram, India

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus 2 (PCV2) are among one of the most prevalent and important infectious agents, which cause a severe economic impact on pig farming worldwide. Circulation of these viruses together in the same pig population may lead to the syndemic infection with altered pathogenicity and thereby pathology and clinical manifestation of the diseases. The present study evaluated syndemic infection between PRRSV and PCV2 in the pig population reared in the backyard farms in Mizoram state of India. The syndemicity between PRRSV and PCV2 was confirmed by clinico-pathological studies followed by simultaneous detection of both the viruses in tissue samples by polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR), immunohisto-chemistry and SYBR green-based real-time PCR / RT- PCR. The syndemicity resulted to a more severe respiratory disease in affected pigs. Pathological studies on affected pigs revealed a combined picture of hemorrhagic lesions with lymphadenopathy. The SYBR green-based absolute quantification assay estimated the PCV2 load in more quantities than that of PRRSV in all the tissues. The highest PRRSV load was detected in the lungs, while the highest PCV2 load was detected in mesenteric and inguinal lymph nodes. The co-circulation of different pathogenic viruses and their persistent infections in a population is always a matter of great concern to pig producers across the world. The present findings highlighted the similar situation with syndemic infection of PRRSV with PCV2 for the first time in Mizoram, India, that caused severe respiratory disease in affected pigs.

Introduction

Pig rearing is one of the fastest growing livestock sectors worldwide that contributes greatly to the economy of many countries. The huge economic potentials of piggery business may suffer from myriads of problems orchestrated by diseases that have the capacity to decimate herds. Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically devastating diseases for the swine industry worldwide caused by PRRS virus (PRRSV).[1] The disease is clinically characterized by reproductive failure characterized by weak neonatal piglets, abortion, stillbirths and mummified fetuses primarily in sows and respiratory illness and high mortality in pigs of all ages.[1] The PRRSV is an enveloped, single stranded, positive sense RNA virus of the order *Nidovirales* family *Arteriviridae* and genus *Betaarterivirus*.[2] The PRRSV is divided into two distinct species namely *Beta-arterivirus suid*-1 (PRRSV-1) and *Betaarterivirus suid*-2 (PRRSV-2) which have a great degree of genetic diversity sharing only 55.00 - 70.00% nucleotide identity.[3,4] The PRRS virion is roughly spherical, 50.00 - 60.00 nm in diameter with an approximately 15.00 kb genome with a 5' cap and a 3' poly A tail. Except for the 5'and 3'untranslated regions at both ends, the PRRSV genome contains at least 11 known open reading frames (ORFs).[5-9]

Porcine circovirus (PCV) 2 is another economically important virus associated with a group of porcine disease conditions that includes post weaning multi-systemic wasting syndrome, porcine dermatitis and nephropathy syndrome, porcine respiratory disease complex, congenital tremors, reproductive failure and more recently, a novel peracute syndrome described as acute pulmonary edema collectively called as PCV associated diseases (PCVAD).[10,11]

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The PCV2 belongs to the family *circoviridae*, genus *circovirus*, and is a small, non-enveloped, isometric virus containing a covalently closed, circular, single stranded DNA genome of 17.00 nm diameter.[12,13] The genome of PCV2 is approximately of 1.70 kb, ambisense and codes for at least six ORFs *i.e.,* ORF1-6. Within the genus circovirus, PCV2 is closely related to non-pathogenic PCV1.[14] The greatest genomic difference between PCV1 and PCV2 are found within ORF3. Based on complete genome analysis or the degree of genetic variation in ORF2, PCV2 strains are classified into at least eight genotypes, PCV2a to PCV2h, with varying prevalence and clinical impact.[15-17]

In India, both PRRS and PCV2 infection among pigs were established very recently.[4,6,18] The first PRRS outbreak in India was recorded during the year 2013 in an epizootic form which was later characterized as the highly pathogenic PRRSV. Since the first outbreak, the piggery sector of Mizoram has been devastated by several major subsequent outbreaks of the disease and is now established as endemic in the pig population of Mizoram.[6,19,20]

The Mizoram state of India, surrounded by international border that facilitates the unrestricted movement of pigs across the vast porous neighbouring border, with other contributing factors like inaccessibility of sufficient doses of vaccine, inadequate facility for timely diagnosis and lack of public awareness have resulted establishing an endemic status for PRRSV and PCV2. The current prevailing situation of co-circulating these two important viruses has increased the possibility for syndemicity between them in the pig population of Mizoram. The present study was envisaged to document the co-infection status of PRRSV and PCV2, two most prevalent viruses in pig population of Mizoram and their pathological manifestations and diagnosis.

Materials and Methods

Study area and sample collection. Tissue samples from a total number of 121 post-mortem cases of different age group of pigs, from 14 organized and 68 backyard commercial herds comprising of total population of 861 pigs, were investigated during the period from August, 2018 to July, 2021. Gross pathological alterations of various organs were recorded and representative tissue samples from lungs, heart, tonsil, lymph nodes, spleen, liver, kidneys and intestine were collected from all the suspected cases with clinical signs suspected of PRRS and PCVAD. Tissue samples were fixed in 10.00% buffered formalin for routine histopathological studies and immunohistochemistry (IHC) and also at – 80.00 ˚C for confirmatory molecular diagnosis. Formalin fixed tissues were subjected to histopathological processing and stained with Hematoxylin and Eosin.[21] Stained individual sections were microscopically examined and the histopathological changes were recorded.

Detection of viral aetiology by polymerase chain reaction (PCR) / reverse transcription-PCR (RT-PCR). All the tissue samples were tested against PRRSV, classical swine fever virus (CSFV), ASFV, PCV2, and PCV3 by using PCR and RT-PCR. For RT-PCR, total RNA was extracted from tissue using Trizol method (Sigma-Aldrich, St. Louis, USA). Reverse transcription of total RNA into cDNA was carried out using a cDNA synthesis kit (Fermentas Life Sciences, Waltham, USA) following the manufacturer's instructions. Previously described primer set, PRRSC– F: CCAGCCAGTCAATCARCTGTG and PRRSC–R: GCGAATCAG GCGCACWGTATG that amplifying a 300 bp fragment of *ORF7* gene of both PRRSV - 1 and PRRSV- 2 was used for detection of PRRSV.[22] To detect ASFV, PCV2 and PCV3, total DNA was extracted from representative tissue samples by using the phenol-chloroform-isoamyl alcohol method and extracted DNA was quantified in Biophotometerplus™ (Eppendorf, Hamburg, Germany). The primer set PCV-L F: 5′ - TAGGTTAGGGCTGTGGCCTT-3′ and PCV-L R: 5′-CCGC ACCTTCGGATATACCG-3′ that amplifies a 263 bp fragment of *ORF-2* gene was used for initial detection of PCV2.[23] All the samples were also tested to rule out the involvement of CSFV, African swine fever virus and PCV3.[24-26]

Immunohistochemistry (IHC). The IHC for detection of PRRSV and PCV2 antigen was carried out with formalin fixed paraffin embedded tissue sections from the tissue samples (lymph node, lung and spleen) confirmed as positive for both PRRSV and PCV2 by PCR and RT-PCR. Four serial sections were prepared from each tissue to use one set for routine histopathology and the others processed for IHC. For IHC tissue sections were taken into amino-propyltriethoxysilane (Sigma-Aldrich) coated glass slides. After de-paraffinization and rehydration, sections were treated with 0.50 mg mL^{-1} proteinase K (Bangalore Genei, Bengaluru, India) for 10 min followed by quenching of endogenous peroxidase activity with addition of 3.00% H_2O_2 in methanol for 15 min at room temperature (RT: 25.00 ˚C). Normal goat serum (5.00%) was used as a blocking agent for 30 min at RT followed by a 5 min rinse in phosphate-buffered saline. The PRRSV polyclonal antibody (Bioss Antibodies, Woburn, USA) for detection of PRRSV antigen and PCV2 capsid protein polyclonal antibody (Bioss Antibodies) for detection of PCV2 were used as primary antibody, respectively. Sections were then treated with peroxidase HRPO-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch, West Grove, USA), for 30 min at RT. The slides were then rinsed twice in phosphate-buffered saline and incubated with diaminobenzidine peroxidase substrate (Vector Lab, Newark, USA) for visualization of antigen-antibody complexes. Sections were counter stained with Mayer's Hematoxylin (Sigma-Aldrich), dehydrated through graded alcohols, cleared into xylene and examined by conventional light microscopy. Positive controls and negative controls were included in each IHC study.

Absolute quantification of viral load The syndemic cases confirmed by detection of both PRRSV with PCV2 by RT-PCR and PCR, respectively, were selected for estimation of viral load in different organs. A SYBR green-based absolute quantification assay was performed using Quanti Nova SYBR green-basedPCR Kit (Quiagen, Hilden, Germany). The in-house developed primer set TGPRRS-F and TGPRRS-R targeting the *ORF7* gene of PRRSV and previously published primer sets PCV-F and PCV-L[23] targeting the *ORF2* gene of PCV2 were used for performing absolute qPCR assay using Rotor Gene 3000 Real-Time PCR instrument (Qiagen). The two gene fragments were cloned using pTZ5R/T vector. Further, plasmids were extracted (Gene JET Plasmid Miniprep Kit; Thermo Scientific, Waltham, USA) and concentration was determined using Biophotometerplus™ (Eppendorf). The plasmid concentration was converted to copy numbers using online software (http://www.scienceprimer.com/copy-number-calculator-for-realtime-pcr) to determine the exact DNA molecules. The quantified plasmids were subjected to 10-fold serial dilution to get standards in the range of 10^9 to 10^1 and the standard curve was prepared. Absolute real time quantification assay of tissue samples from different organs belonging to the syndemic cases of PRRSV with PCV2 was performed along with the standards. The threshold cycle (Ct) values of test samples were compared against the Ct values of standards to determine the viral load in different tissues.

Results

Clinical and pathological findings. Syndemic infection of PRRSV with PCV2 in field condition was detected in six backyard pig farms located in Aizawl district of Mizoram. Sudden onset of severe depression, anorexia and respiratory distress characterized by forceful respiration, secretion of catarrhal nasal discharge with high fever (103 - 106 ˚C) were the first clinical signs observed in the affected pigs. Subsequently cutaneous hyperemia was developed, particularly at the tip of the ear, ventral surface of the abdomen and extremities of legs.

Necropsy examination in seven of the dead pigs revealed mild to moderate ascites, hydrothorax and hydropericardium with petechial hemorrhages on epicardium. Lungs were edematous, non-collapsing, congested with areas of ecchymotic hemorrhages and cranio-ventral consolidation of apical lobe (Fig. 1A). Liver appeared icteric with numerous whitish pale necrotic foci. Kidneys were swollen and congested. Enlarged, congested to mottled and hemorrhagic appearance were notable in all the superficial to regional lymph nodes. Spleen showed infarction at the edges with numerous petechiations on the surface (Fig. 1B). Hemorrhagic gastritis with ulceration, hemorrhagic enteritis and congested palatine tonsils with petechiation were also observed in the affected pigs.

Histopathological examination of lungs showed hemorrhagic interstitial pneumonia characterized by infiltration of mononuclear inflammatory cells, oedema and severely congested alveolar capillaries leading to thickening of alveolar wall (Fig. 1C). Lumen of bronchioles and alveoli were filled with serous exudates, desquamated epithelial cells, mononuclear cells and necrotic debris. Bronchitis with hyperplastic bronchiolar epithelium, reactive and diffuse proliferation of bronchial associated lymphoid tissue in peribronchiolar area was also observed in lungs (Fig. 1D). Heart displayed degeneration of cardiac muscle fibres, hemorrhage and infiltration of inflammatory cells in myocardium. Severe degenerative and necrotic changes in renal tubular epithelial cells, diffuse hemorrhages in interstitium, vasculitis and intravascular coagulopathy indicated by hyalinised and homogenized clot surrounded by red blood cells in blood vessels and congested glomerular tuft were observed in kidney. Extensive necrotic changes with complete loss of hepatocytes in focal areas infiltrated by mononuclear cells and disorganization of hepatic cords were observed in liver (Fig. 1E). Severe lymphoid depletion with infiltration of histiocytes, necrotic parafollicular/cortical area and vasculitis were observed in lymphoid organs (lymph nodes, spleen and tonsil). Microscopically, intestine revealed total loss of villus along with necrosis of crypt cells and thickening of lamina propria due to severe infiltration of mononuclear cells.

Confirmatory diagnosis by PCR / RT-PCR and IHC. Tissue samples from total number of 121suspected cases were tested by PCR / RT-PCR, for PPRSV, PCV2, PCV3, CSFV and ASFV. A total number of 11 necropsy cases from 6 different backyard pig farms were tested positive for both PRRSV and PCV2. Additionally, a total number of 15 cases from other farms were tested positive only for PRRS and five cases were tested only for PCV2. Amplification of expected product of about 300 bp fragments of *ORF7* gene[22] of PRRSV and 263 bp fragment of *ORF2* gene of PCV2[23] were yielded simultaneously in all the 11 necropsy cases (Fig. 2). All the samples were tested negative for ASF, PCV3 and CSFV. Serial tissue section (Lungs, lymph node and spleen) of PRRSV and PCV2 co-infected PCR positive cases were selected for simultaneous in situ demonstration of PRRSV and PCV2 antigen by immuno-histochemical staining. Strong positive signals for PRRSV antigen were detected in infiltrating mononuclear cells in alveolar space, interstitium and lumen of bronchioles of lung (Fig. 1F and G). The PCV2 antigen was mostly detected in bronchiolar epithelial cells and also in infiltrating mononuclear cells in alveolar air space (Fig. 1H and I) with moderate to strong positive immunostaining. Similarly, intense positive immunostaining was demonstrated for both PRRSV and PCV2 antigen in infiltrating macrophages within the depleted follicle of spleen and in lymph node. This further established the

outbreaks of PRRS and PCV2 syndemic infection in field condition among pig population of Mizoram.

Absolute quantification assay. Presence of PRRSV and PCV2 in the same tissue samples was also confirmed using SYBR green-based real time PCR. The viral load of both the PRRSV and PCV2 in different tissues was quantified. Standard curves were prepared using the Ct values of standards (ranging from 10^8 to 10^4 copies *per* 10 µL reaction) for the calculation of both PRRSV and PCV2 viral loads in different tissues. The Ct values of standards ranged from 10.68 to 25.23 for PCV2 and 14.99 to 38.03 for PRRSV. The presence of viral copies in different organs was compared to the standard curve and the exact copy numbers were calculated and expressed as given in the Table 1. The highest PCV2 mean copy number was detected in mesenteric lymph node (2.78×10^7 copies μL^{-1}) and inguinal lymph node (2.73×10^7 copies μL^{-1}), and the least was observed in heart (3.45×10^6 copies μL^{-1}). Whereas, the highest copy number for PRRSV was observed in lungs (4.12×10^5 copies μL^{-1}) followed by bronchial lymph node (1.47×10^5 copies μL^{-1}) and the least was observed in heart (8.62×10^3 copies μL^{-1}), (Table1).

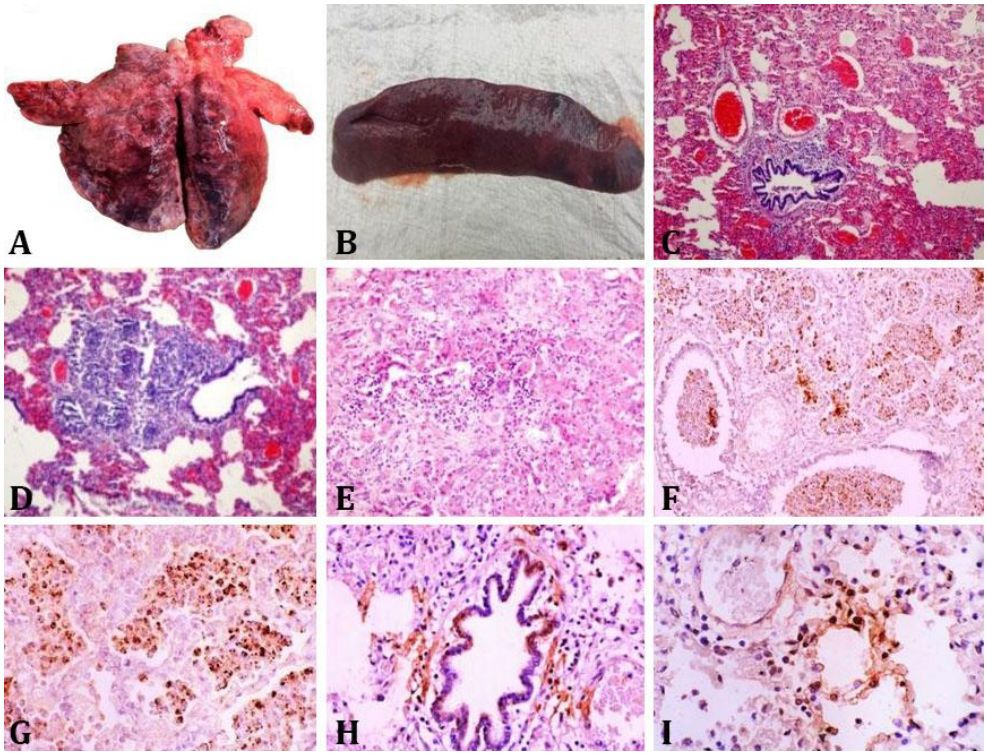

Fig 1. A) Lung: Non-collapsing, hemorrhagic lung with areas of consolidation. **B)** Spleen: Infracted areas and petechiation on the surface of spleen. **C)** Lung: Thickening of interstitium due to infiltration of mononuclear cells, edema, hemorrhages and vascular congestion. (Hematoxylin and Eosin, 200×). **D)** Lung: Bronchial associated lymphoid tissue proliferation around peribronchiolar region. (Hematoxylin and Eosin, 200×). **E)** Liver: Extensive necrosis, (Hematoxylin and Eosin, 200×). **F and G)** Lung: Co-infection porcine reproductive and espiratory syndrome virus (PRRSV) with porcine circovirus 2 (PCV2): PRRSV antigen in nuclei of infiltrating mononuclear cells in interstitium, alveolar space and bronchiolar lumen. Immunohistochemistry: Diaminobenzidine substrate, Mayer's Haematoxylin counter stain. (F 400× and G 600×). **H and I)** Lung: co-infection PRRSV with PCV2. PCV2 antigen in nuclei/cytoplasm of infiltrating mononuclear cells in alveolar space, interstitium and bronchiolar epithelial cells. Immunohistochemistry: (Diaminobenzidine substrates, Mayer's Hematoxylin counter stain. H, 400 × and I, 600×).

Table 1. Viral load in different tissues from pig co-infected with porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus 2 (PCV2) expressed as copies μL^{-1}.

Organs	PCV2 concentration	PRRSV concentration
Lung	7,905,290	412,313
Kidney	6,904,244	40,225
Liver	17,463,802	38,468
Spleen	13,045,206	48,608
Heart	3,452,793	8,627
Mesenteric lymph node	27,806,457	136,563
Inguinal lymph node	27,386,639	93,216
Bronchial lymph node	18,951,825	147,853
Gastrohepatic lymph node	13,288,068	49,371

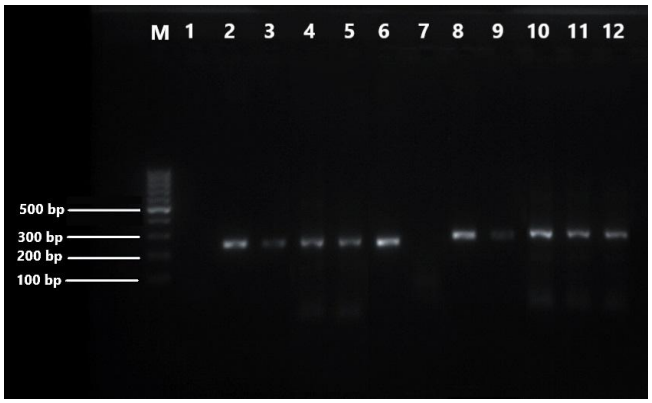

Fig. 2. Agarose gel electrophoresis stained with ethidium bromide. Lane M: 100 bp DNA marker; Lane 1: No template control (NTC), Lanes 2 - 6: Spleen, mesenteric lymph node, inguinal lymph node, bronchial lymph node, and lungs issues showing the 263 bp fragment of porcine circovirus 2. Lane 7: NTC, Lanes 8-12: Spleen, mesenteric lymph node, inguinal lymph node, bronchial lymph node and lungs showing the 300 bp fragment of porcine reproductive and respiratory syndrome virus from necropsy No. 122-A/21.

Discussion

Porcine reproductive and respiratory syndrome virus and PCV2 associated diseases are two of the important viral diseases of swine, which are frequently encountered and are mainly responsible for mortality in swine population of Mizoram.[4,6,9,18,19] In the present study, co circulation of both the viruses in the affected pig farms might have led to the syndemicity between PRRSV with PCV2 that resulted 100% mortality in the affected six backyard farms within a period of 1 to 2 weeks of time.

Pathological studies on affected pigs revealed a combined picture of hemorrhagic lesions associated with vasculitis and lymphadenopathy characteristic to PRRS and PCVAD, respectively. Hemorrhagic, non-collapsing and consolidated lungs icteric liver, enlarged and hemorrhagic lymphadenitis and spleen with areas of infarction were conspicuous changes noticed in the pigs died of the syndemic infection. Microscopically, focal necrosis and loss of hepatic architecture in liver, hemorrhagic myocardium, tubular necrosis in kidney and severe lymphoid depletion with hemorrhages in the inter-follicular region in lymphoid organs were predominantly observed. Overall, more aggravated form of disease progression and pathological changes were observed in all the affected pigs. The association of concurrent PCV2 - PRRSV infection with significant increase in severity of disease has been described earlier in both experimental and field cases.[27-30] The degrees of severity of pneumonic lesions have also been described to be more in syndemic infection than any singular infection of PRRSV or PCV2.[31,32]

The PRRSV and PCV2, both can replicate in monocyte/ macrophage-lineage cells including alveolar macrophages in the lymph nodes and tissues.[33,34] Both the viruses may persist for prolonged time in host lymphoid organs associated with persistence infection.[35,36] The IHC has demonstrated PRRSV and PCV2 antigen in the nuclei/ cytoplasm of infiltrating mononuclear cells in alveolar space/intestitium, bronchiolar epithelial cells of lungs and infiltrating histiocytes within the depleted follicle of spleen and lymph nodes.[36]

The SYBR green-based absolute quantification assay has estimated the PCV2 viral load more than that of PRRSV in all the tissues. As expected, the highest PRRSV load was detected in lungs being the target organ for the virus, while highest PCV2 load was detected in mesenteric and inguinal lymph nodes. The hallmark lesion associated with PCV2 infection was lymphadenopathy which correlated with lymphoid depletion at the cellular level.[36,37] This might be the probable reason behind the presence of the highest PCV2 viral load in lymphoid tissue.[37-39]

In recent times, co-infection of PRRSV - PCV2 has become a great concern as PRRSV potentiates PCVAD manifestation and increases disease severity compared to pigs infected with PCV2 alone.[39,40] Although PCVAD is a multifactorial disease, PCV2 alone is sufficient to develop clinical PCVAD, however, may not always lead to a clinical form of disease, causing disease in varying percentages of animals within a group while the rest of the herd typically might not display clinical signs.[40] However, whenever PCV2 is present in conjunction with other pathogens, it always leads to more severe form of the disease and is characterized by high morbidity and mortality in the affected group.[41,42]

The exact mechanism of synergistic effect of PRRSV when co-infected with PCV2 is not well understood. Both PCV2 and PRRSV viruses have affinity towards Pulmonary alveolar macrophages (PAMs) and both are capable of infecting PAMs.[34,42] When PRRSV infects and starts replicating inside PAMs, it leads to modification of macrophage functions and causes immune deregulation which renders the host unable to clear concurrent infections. Moreover, PCV2 decreases the efficiency of swine alveolar macrophages by inhibiting production of oxygen-free radicals and hydrogen peroxide needed for microbiocidal activity.[41,43,44] Thus, PRRSV potentiates and helps PCV2 replicate inside host cells which results in increased amounts of PCV2 DNA in co-infected pigs and increased severity of PCVAD.[45,46] Experimental studies have also suggested that the pathogenicity may also depends upon which is the virus that infected the individual earlier.[39] Thus, the synergistic effect of PRRSV and PCV2 in a syndemic infection is much more complex that require further research for proper understanding.

The syndemicity of PRRSV with PCV2 in the present study recorded more systemic involvement with pronounced clinical disease characterized by severe respiratory distress, diarrhoea, extensive pathological changes in lungs, spleen, liver, kidney and generalized

lymphadenopathies. All the pigs eventually died in the affected farms suggesting an aggravated form of the disease compared to PRRSV and PCV2 alone.

In conclusion, the present study evaluated the co-infection status of two most important viruses which were prevalent in Mizoram. The exact mechanism of synergistic effect of PRRSV and PCV2 on affected pigs was not well understood. However different field and experimental studies including the present study have established that such infections always led to more-severe clinical signs and lesions resulting to higher mortality. The co-circulation of different pathogenic viruses and persistent infections in a population are matter of great concern and pose a major threat to pig producers across the world. In such situation, the approach for control and prevention against the prevailing viral diseases may demand a holistic approach rather than existing strategy of focusing on individual diseases.

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Conflict of interest

The authors declare no competing interests.

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