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Molecular Diagnosis of Porcine Reproductive and Respiratory Syndrome Outbreaks in Pigs of Mizoram State

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ABSTARCT

Background: Porcine reproductive and respiratory syndrome (PRRS) is considered as an economically important viral disease of pigs worldwide. India has reported the first outbreak of PRRS in the pig population of Mizoram state to the Office International des Epizooties (OIE) on the 26 June, 2013. Although the disease appeared to be contained in the Mizoram state, several severe outbreaks of the disease has been reported in subsequent years. The present study has determined the current status of porcine reproductive and respiratory syndrome in the pig population of Mizoram state and its diagnosis by molecular and immunohistochemistry technique.

Methods: A total number of 365 pigs of different age groups from 5 organized and 35 backyard farms located in different districts of Mizoram were studied. A detailed necropsy was conducted on 88 dead pigs. Gross lesions were recorded and representative tissue samples were collected for histopatholgical, immunohistochemical studies and preserved at -80°C for molecular diagnosis.

Result: Respiratory signs characterized by difficulty in breathing accompanied with high fever were the clinical signs observed in affected pigs. Severe haemorrhages in lungs with interstitial pneumonia, haemorrhagic lymphadenitis with lymphoid depletion in lymphoid organs were the most commonly observed lesions followed by haemorrhagic myocarditis and nephritis. Reverse transcription PCR targeting 300 bp fragments of ORF7 gene and real-time PCR targeting 152 bp fragments of ORF7 gene of PRRSV from the tissue extracted RNA (lung, lymph node, spleen) confirmed the outbreaks of PRRSV in different backyard and organized swine herds. The PRRSV antigen was demonstrated by immunohistochemistry in alveolar macrophages in lungs and infiltrating macrophages in spleen and lymph nodes tissues respectively.

Key words: Immunohistochemistry, Mizoram, ORF7, PRRS, Real-time PCR, RT-PCR.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a devastating infectious swine disease and has become a major threat to the swine industry worldwide. The disease is characterized by reproductive failure in sows and respiratory tract illness in pigs of all ages. The causative organism of PRRS, porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped, single-stranded; positive-sense RNA virus belongs to the order of Nidovirales, family *Arteriviridae* and genus *Betaarterivirus* (Brinton *et al*., 2021). The whole viral genome of PRRSV is approximately 15 kb in length and consists of a 5′-untranslated region (UTR), nine partially overlapping open reading frames (ORFs), ORF1a, ORF1b, ORF2a, ORF2b and ORF3 to ORF7, followed by a 3′-UTR and a poly (A) tail (Dea *et al*., 2000; Meulenberg, 2000).The disease was first documented in 1987 in the USA and became a pandemic in North America, Europe and Asia in the succeeding years (Benfield *et al*., 1992; Botner *et al*., 1994).

There are two species of *Betaarterivirus*, namely *Betaarterivirus suid- 1* (PRRSV -1) and *Betaarterivirus suid - 2* (PRRSV-2) with nucleotide similarity of only 55%-70% (Brinton *et al*., 2021; Liu *et al*., 2020). Highly pathogenic-PRRSV (HP-PRRSV), a novel variant of PRRSV-2 that emerged in China in 2006 caused severe disease in Asia (Tian *et al*., 2007). The epidemiology and genetic diversity of PRRSV is constantly changing as a result of high mutational rate of PRRSV, which is an eminent feature of many RNA viruses. In the recent past the swine herds of three bordering countries of India, namely China, Myanmar and Bhutan were severely hit by outbreaks of highly pathogenic - PRRS (HP-PRRS) (Shi *et al*., 2010; Yu *et al*., 2012). In India, the first outbreak of PRRS was reported from Mizoram and subsequently reported to the Office International des Epizooties (OIE) by the Govt. of India on 26th of June 2013. Genetic analysis showed that the Indian strain of PRRSV is identified as genotype 2, which is closely related to HP-PRRSV of China (Rajkhowa *et al*., 2021; Rajkhowa *et al*., 2015; Gogoi *et al*., 2017). The first outbreak of PRRS in

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Mizoram, India occurred in an epizootic form affecting all age groups including the pregnant sows with high morbidity and mortality that resulted in huge economic losses to the pig farmers (Rajkhowa *et al*., 2015). Since the first outbreak in 2013, the disease is now established as an endemic in pig population of Mizoram resulting in regular sporadic outbreaks in the state. The present study has enlightened the dynamics of PRRS in pig population of Mizoram in the recent years.

MATERIALS AND METHODS

Sample collection and study area

During the period from 2018 April to January, 2021, a total of 5 organized and 35 backyard farms that comprised a total of 365 pigs located in different districts of Mizoram were visited following complains of acute respiratory diseases with mortality in affected pigs. A total of 151 clinically affected pigs were examined at the farm premises and detailed post mortem examination was conducted in 88 pigs that died (Table 1). Gross lesions were recorded and representative tissue samples from lungs, spleen, kidneys, liver, tonsil and lymph nodes were collected and preserved in 10% buffered formalin for histopathological and immunohistochemical studies and also at -80°C for molecular studies. Formalin fixed (48 hrs) tissues were subjected to histopathological processing and staining following standard method (Bancroft and Gamble, 2008). Hematoxylin and eosin stained individual sections of 5 µm thickness were examined using Olympus trinocular research microscope fitted with digital camera and the histopathological changes were recorded.

Table 1: Details of pig population studied.

Details of pig population studied					PCR results		
Farm details (type)	Total farm strength	Breed	Age group (months)	Total pig died/ necropsy conducted	PRRS	CSF	PCVAD
Organized	37	LWY	3	6	+ve	-ve	-ve
Backyard	7	CB	4	3	+ve	-ve	-ve
Backyard	5	Zovawk	3	1	-ve	-ve	-ve
Organized	31	CB	5	4	+ve	-ve	-ve
Backyard	7	LWY	4-5	2	+ve	-ve	-ve
Backyard	11	CB	5	3	+ve	-ve	-ve
Backyard	5	LWY	4	2	-ve	-ve	-ve
Backyard	10	LWY	1.5	1	+ve	-ve	-ve
Backyard	5	LWY	2.5	2	-ve	-ve	-ve
Backyard	9	Zovawk	2	5	-ve	-ve	-ve
Backyard	7	CB	3	3	-ve	-ve	-ve
Backyard	8	CB	3.2	2	-ve	-ve	-ve
Backyard	12	LWY	7	4	+ve	-ve	-ve
Backyard	6	LWY	3	3	-ve	-ve	-ve
Backyard	8	LWY	1 yrs.	2	+ve	-ve	-ve
Backyard	7	LWY	3	1	-ve	-ve	-ve
Backyard	9	CB	5	4	-ve	-ve	-ve
Backyard	11	LWY	6	3	+ve	-ve	-ve
Backyard	5	LWY	2	2	+ve	-ve	-ve
Backyard	7	LWY	4	2	+ve	-ve	-ve
Backyard	9	CB	3	3	-ve	-ve	-ve
Organized	23	CB	5-6	3	-ve	-ve	-ve
Backyard	8	CB	3-4	2	+ve	-ve	-ve
Backyard	5	CB	3	1	-ve	-ve	-ve
Backyard	6	Zovawk	6	2	-ve	-ve	-ve
Backyard	8	CB	5	3	+ve	-ve	-ve
Backyard	7	LWY	3	1	-ve	-ve	-ve
Backyard	5	LWY	4	2	+ve	-ve	-ve
Backyard	6	CB	3-4	2	+ve	-ve	-ve
Backyard	12	CB	2-3	2	-ve	-ve	-ve
Backyard	6	CB	5-6	3	-ve	-ve	-ve
Backyard	7	CB	3-4	2	-ve	-ve	-ve
Backyard	8	CB	1 yrs.	2	+ve	-ve	-ve
Backyard	5	CB	3	2	-ve	-ve	-ve
Backyard	12	Zovawk	6	3	+ve	-ve	-ve

Polymerase chain reaction (PCR)/reverse transcription-PCR (RT-PCR)

For reverse transcription-PCR, total RNA was extracted from tissue samples (lungs, spleen and lymph nodes) by using Trizol method (Sigma-Aldrich, St. Louis, MO, USA). Reverse transcription of total RNA into cDNA was carried out by using cDNA synthesis kit (Fermentas Life Sciences, Canada) according to the manufacturer's protocols. All the tissue samples were tested by reverse transcription - PCR for initial detection of PRRSV by using previously described conditions with a degenerate primer set, PRRSC-F: CCAGCCAGTC AATCARCTGTG and PRRSC-R: GCGAATCAGGCGC ACWGTATG that amplify about 300 bp fragment of ORF7 gene of both PRRSV - 1 and PRRSV- 2 (Toplak *et al*., 2012). For differential diagnosis all the samples were also tested for classical swine fever virus (CSFV) targeting the genomic region that encodes the 5′ NTR (NTR-F: CTAGCCATGCC CWYAGTAGG and NTR-R: CAGCTTCARYGTTGATTGT) (Greiser-Wilke *et al*., 1998) and Porcine circo virus 2 (PCV2) targeting 263 bp region of ORF 2 gene (PCVL-F: 5′ TAGGTTAGGGCTGTGGCCTT-3′ and PCVL-R: R-5′CC GCACCTTCGGATATACTG3') (Larochelle *et al*., 1999).

Real-time PCR detection of PRRSV

Representative cDNA samples from PRRSV positive cases (previously diagnosed with RT-PCR) were further subjected for Sybr-green real-time PCR assay targeting 152 bp fragments of ORF7 gene by using in house developed primer set, TGPRRS-F:5′-ATCGCCCAACAAAACCAGTC-3′ and TGPRRS-R:5′- TGGATCGACGACAGACACAA-3′ with no template control (NTC) and positive control. After initial denaturation of 95°C for 5 min, 45 amplification cycles were conducted with each cycle consisting of 95°C for 15 sec (denaturation), optimized annealing temperature of 60°C for 15 sec and 72°C for 15 sec (extension). On completion of 45 cycles a final extension was given for 15 sec at 72°C. Following the amplification, melt curve analysis was performed to measure the specificity of the product. Temperature profile for melt curve included ramping of temperature from 65°C to 95°C with 1°C increase and 5 sec hold in each step.

Immunohistochemistry (Indirect immunoperoxidase test)

For visualization of PRRSV antigen, indirect peroxidase technique (IPT) was done on formalin fixed paraffin embedded lymph node, lung and spleen tissue sections. Sections were cut at thickness of 5 µm thickness and sections were taken into APES (Aminopropyltriethoxysilane) coated glass slides. Slides were deparaffinised in xylene (twice for 5 min), then rehydrated in descending series of alcohol [absolute alcohol (twice for 3 min), 95% alcohol (twice for 5 min), 80% alcohol (3 min), 70% alcohol (3 min)] and finally washed under running tap water for 1 min. Antigen unmasking was done by treatment with Proteinase K (0.5 mg/ml) (Bangalore Genei) for 10 minutes and quenching of endogenous peroxidase activity was done by addition of 3% H_2O_2 in methanol for 15 minutes at room temperature. All tissue sections were incubated with 5% normal goat serum for 30 minutes at room temperature (25°C) to block the non-specific binding of the antibodies. Primary antibody (PRRSV polyclonal antibody, product code- bs-10043R, Bioss Antibodies) were applied and kept for overnight in 4°C. Peroxidase HRPO -Conjugated Affinipure Goat Anti-Rabbit IgG (Product code-111-035-003, Jakson Immunoresearch Lab) was applied as secondary antibody for 30 min at room temperature. For visualization of immunoreaction DAB (Victor Lab) substrate was added to tissue section and finally counter stained with Mayer's haematoxylin. Washing with PBS was carried out in between every step. Slides were mounted in DPX and examined under trinocular research microscope fitted with digital camera (Olympus) and compared with positive and negative control.

RESULTS AND DISCUSSION

Clinical findings

The outbreaks of PRRS were reported from both organized and backyard farm investigated during the study period. The affected pigs were depressed, anorexic and suffered from moderate to high fever (103-105°C). Clinical signs were detected in young post-weaned grower pigs (3-7 months age) and no abortion or PRRS associated reproductive failure were documented in the present study. The respiratory signs were predominant, characterized by forceful respiration or open mouth breathing with occasional catarrhal nasal discharge in severely affected pigs. The affected pigs also exhibited hyperaemic or purplish discolouration of skin mainly at the tip of the ear, ventral abdomen and extremities of leg. Morbidity and mortality rates during the present study recorded were 41.37% and 24.11% respectively.

The consistent characteristic clinical signs observed in affected pigs includes moderate to high fever accompanied with respiratory distress and erythematous skin lesions. Similar findings with severe intensity were described by earlier authors (Rajkhowa *et al*., 2015; Gogoi *et al*., 2017).

Gross, histopathology and immunohistochemistry

Detailed post mortem examination revealed most prominent gross lesions in lungs, spleen and lymph nodes. Lungs were non-collapsible, oedematous, congested with ecchymoses with diffuse involvement (Fig 1A). The cut surface of lungs showed blood mixed frothy fluid. Hydropericardium (30-40 ml of straw colour fluid) and pinpoint to ecchymotic haemorrhages on epicardium of the heart was observed (Fig 1B). Mediastinal, inguinal and mesenteric lymph nodes were enlarged, congested and oedematous. Spleen was congested with areas of infarction and petechiae in focal areas (Fig 1C). Kidneys appeared congested and swollen. Mild congestion of palatine tonsils was noticed in the affected pigs.

Microscopic examination of lung tissue sections revealed severe interstitial pneumonia, congestion and oedema with haemorrhage in interstitum and alveolar space. Interstitial space and alveolar lumen were filled with mononuclear inflammatory cells (Fig 1D). Lumen of bronchioles was filled

with desquamated epithelial cells, mononuclear inflammatory cells and necrotic debris. Degeneration and fragmentation of cardiac myofibres, haemorrhage and mild infiltration of mononuclear inflammatory cells were observed in heart.

Spleen showed sinusoidal congestion, severe lymphoid depletion, extensive haemorrhages, areas of infarction and necrosis in parafollicular area. Palatine tonsil, inguinal, mesenteric and bronchial lymph nodes displayed follicular lymphoid depletion with congestion and oedema in the interfollicular region (Fig 1E). Kidney revealed distended tubular lumen, coagulative necrosis and desquamation of tubular epithelial cells, congestion and focal haemorrhage in cortical area. The gross and histopathological findings observed in the present study are in corresponding with earlier authors and were consistently observed in all the reverse transcription PCR positive PRRS cases with certain degree of variation (Lager and Halbur, 1996; Zimmerman *et al*., 2012; Gogoi *et al*., 2017).

IHC allows localization of virus particles inside infected tissue or cells (Kim *et al*., 2009). Tissue sections from reverse transcription–PCR positive PRRSV cases were selected for localisation of virus antigen *in situ* by immunohistochemistry. Lymph node sections showed intense positive immuno staining of PRRSV antigen mostly in infiltrating macrophages in lymphoid cells of depleted follicles (Fig 1F, 1G). Intense immunopositivity in infiltrating macrophages within the depleted follicle of spleen and in alveolar macrophages of tissue sections from lungs was demonstrated. The demonstration of the PRRSV antigen *in situ* by IHC in formalin-fixed, paraffin embedded tissue sections from lymph nodes; lungs and spleen have further confirmed the diagnosis of PRRS field outbreaks in pig population of Mizoram.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR results

Tissue samples from 88 necropsy cases were tested by RT-PCR targeting ORF-7 gene of PRRSV. Amplification of expected product of about 300bp was observed in total 46 (52.27%) necropsy cases (Fig 2). All the samples were tested negative for CSFV by RT–PCR. Sybr-green real-time PCR

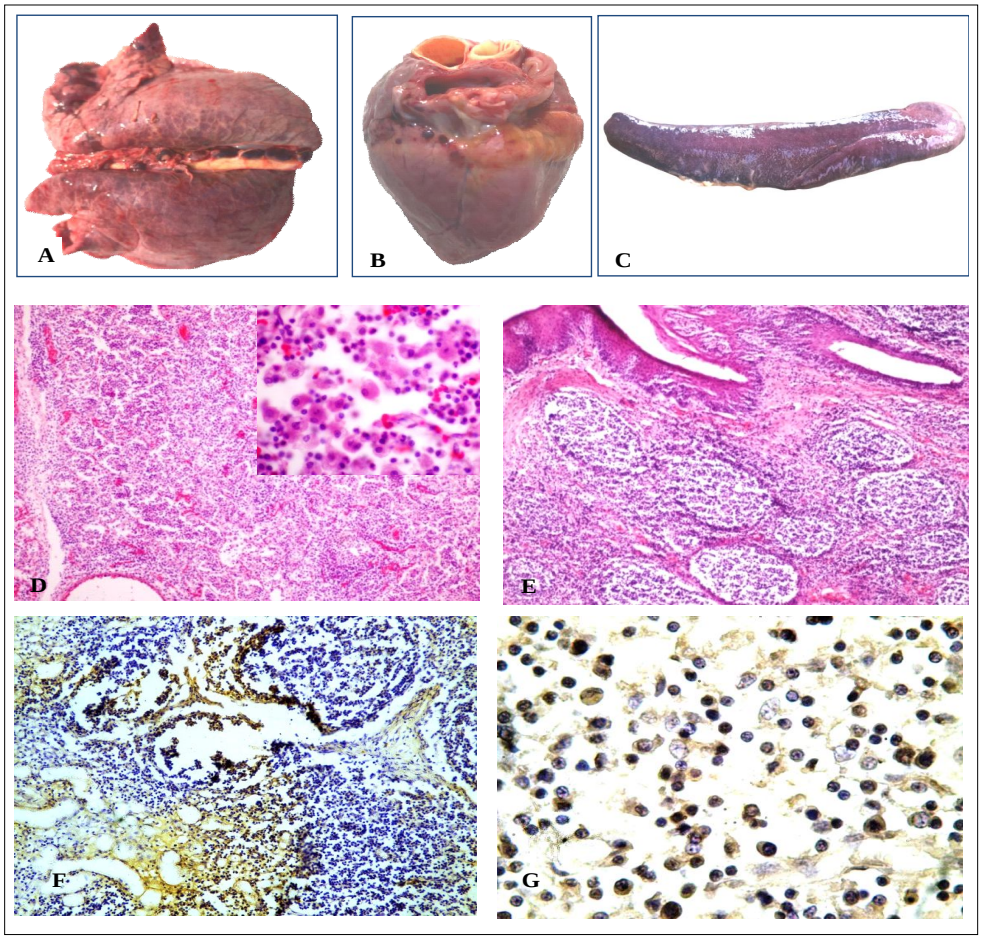

Fig 1: (A) Non-collapseable, congested lung with echymotic haemorrhage. (B) Haemorrhages on epicardium of the heart. (C) Congested and infarcted areas on the surface of the spleen. (D) Lung: Section showing interstitial pneumonia due to extensive infiltration of mononuclear inflammatory cells in interstitum and alveolar lumen along with congestion and oedema. H & E, × 100. (E) Tonsil:Lymphoid depletion and vascular congestion. H & E, × 200. (F & G) Inguinal lymph node(IHC, PRRSV): PRRSV antigen in infiltrating histiocyte or macrophage in depleted lymphoid follicle. DAB substrate, Mayer's haematoxylin counter stain (F. × 200, G. × 600).

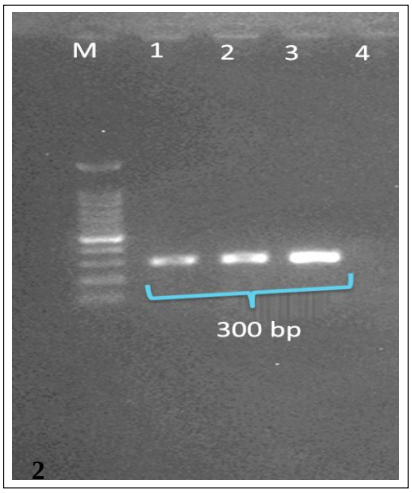

Fig 2: Agarose gel electrophoresis showing 300 bp PCR amplicon from ORF7 gene of PRRSV. (M = Molecular marker, 1 = Lung, 2 = Lymph node, 3 = Positive control PRRSV, 4 = No template control).

assay based diagnosis of test samples with no template control (NTC) and positive control displayed fluorescent curves for the amplification of 152 bp fragment of PRRSV specific ORF7 gene, indicating that all the test samples were positive. Ct values of positive samples were ranging from 23 to 32. Melt curve analysis resulted in a single peak representing constant amplification of the target gene fragment (Fig 3, 4). The field outbreaks of PRRS during the study period were all confirmed by both reverse transcriptase -PCR and Sybr-green real-time PCR assay targeting detection of the ORF7 gene of PRRSV. Similar amplification of specific sequence of PRRSV by RT-PCR using type specific primers was reported previously (Rajkhowa *et al*., 2015; Hopper *et al*., 1992).

Porcine reproductive and respiratory syndrome (PRRS) continues to be a threat for the pig industry worldwide. It is still prevalent in many parts of the world and emergence of new strains, absence of an effective vaccine makes it more difficult to prevent and control. Introduction of novel HP-PRRSV in Asian countries including India, has posed another

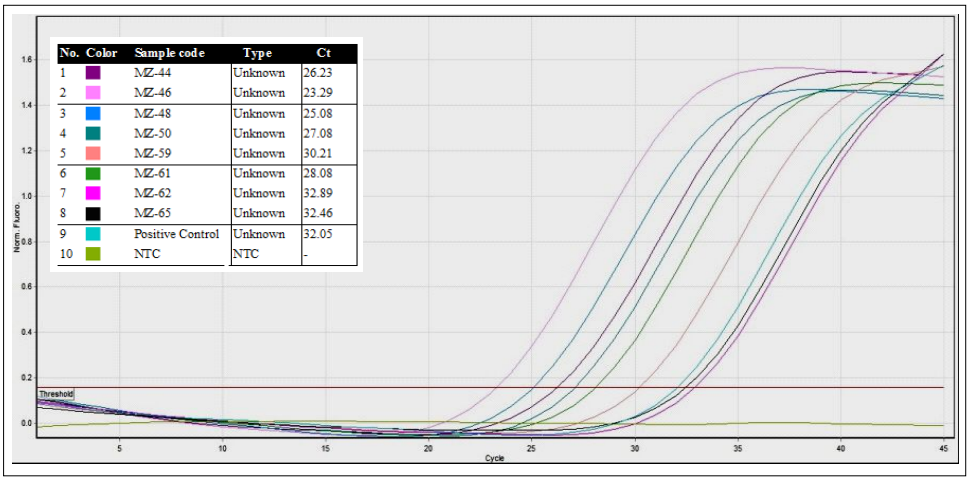

Fig 3: Sybr-green real-time PCR assay-fluorescent curves representing the threshold cycles (Ct) value of PRRSV suspected samples, amplifying 152 bp fragment of ORF7 gene.

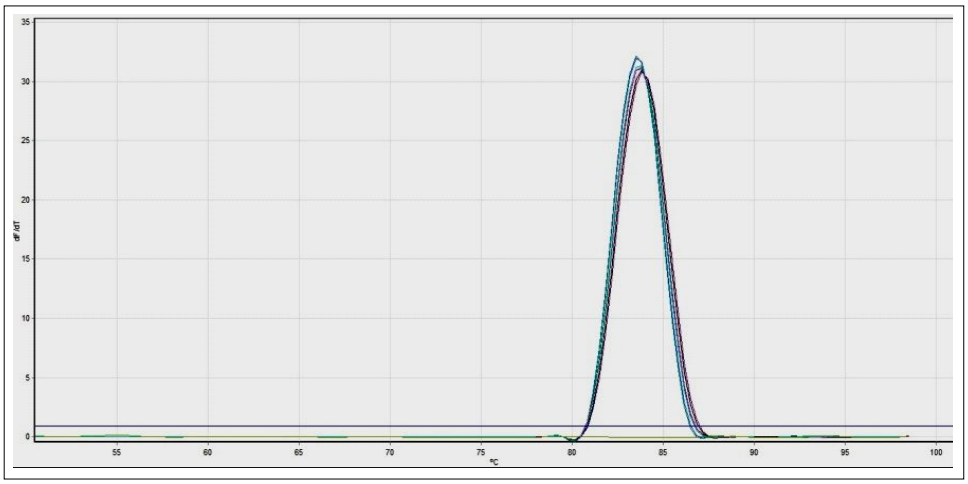

Fig 4: Sybr-green real-time PCR assay for PRRSV-Melt curve analysis of test samples, NTC and positive control.

challenge for the pig farmers in Asia (Rajkhowa *et al*., 2021, Zhou *et al*., 2008).

The first outbreak of the disease in the year 2013 and the subsequent outbreaks in 2015, 2016 and 2018 were recorded in epidemic form with high morbidity and mortality (up to 81% morbidity and 68% mortality) (Rajkhowa *et al*, 2015, Gogoi *et al*., 2017). The present study has recorded the magnitude of the disease in less intensity in terms of morbidity (41.37%) and mortality (24.11%) in affected farms when compared to earlier outbreaks. This may be attributed either to natural herd immunity developed by exposed animals or reduced virulence of the circulating PRRSV strains.

CONCLUSION

Current investigation has established that PRRSV is circulating continuously and causing sporadic outbreaks in susceptible pigs in Mizoram, India. Although the magnitude of the disease is recorded in low intensity compared to earlier outbreaks, the clinical disease in susceptible pigs was always presented in acute form with moderate to severe pathological lesions. The sporadic outbreaks of the disease with varying degrees of clinical signs and pathology have indicated that the disease has established endemic status in the pig population of Mizoram. The current situation demands special attention for control and prevention of the disease. Mizoram shares a completely porous international border with Myanmar and Bangladesh, which allows free movement of animals from both the sides. This has put the state at high risk for transboundary diseases like PRRS in pigs. Considering the facts that PRRSV is genetically diverged and is continuously evolving through very high rate of mutation and recombination, continuous monitoring and surveillance of the disease is paramount for control and containment of PRRS to protect the pig population in India.

Conflict of interest: None.

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