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Pathology of PMWS Associated with Classical Swine Fever Virus, Porcine Parvo Virus and Multidrug Resistant Enteric *Escherichia coli* Infections in an Organized Swine Farm, Rajasthan, India

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ABSTRACT

Background: Postweaning multisystemic wasting syndrome (PMWS) is caused by porcine circovirus type 2 (PCV2). It is a globally emerging clinical condition which affects nursing piglets and growing pigs. PCV2 requires various cofactors for fully development of PMWS. The present report describes the pathology of PMWS associated with classical swine fever virus (CSFV), porcine parvo virus (PPV) and multidrug resistant enteric *Escherichia coli* infections in an organized swine farm, Rajasthan. In India, this is the first report on pathology of PMWS associated with all the three viruses and multidrug resistant enteric colibacillosis.

Methods: Detailed necropsy was conducted on pig carcass submitted to Post-mortem Facility, Division of Pathology, ICAR-Indian Veterinary Research Institute (IVRI), Izatnagar in the year 2017 from an organized swine farm, Rajasthan, India. Heart blood and diarrhoeic intestinal contents were collected aseptically and stored separately at 4°C for bacteriological examination and antibiotic sensitivity test. Tissue samples were collected in sterile container and stored at -40°C for DNA and RNA extraction and polymerase chain reaction (PCR) and also collected in 10% neutral buffered saline for histopathological processing.

Result: The organized farm was having history of 33% (200/600) of mortality among 2 months to 6 months old piglets. Gross lesions were wasting, lymphadenopathy, non-collapsed lungs, hydropericardium, hepatomegaly, icterus, enteritis and pale kidneys, button shaped ulcers in the caecum and colon *etc*. Histopathologically, lymphoid depletion, reticuloendothelial cell proliferation in lymphoid tissue, fibrin thrombi in spleen, severe hepatitis, interstitial pneumonia, interstitial nephritis, severe necrosis in large intestine, meningitis, perivascular cuffing (PVC), gliosis, neuronal degeneration in cerebrum *etc* were observed. Molecular diagnosis and bacteriological examination confirmed presence of PCV2, PPV, CSFV and multidrug resistant *Escherichia coli*.

Key words: CSFV, *Escherichia coli*, Pathology, PCR, PCV2, Piglets, PMWS, PPV.

INTRODUCTION

Pig is one of the key domesticated livestock and it is having significant role towards food security and socioeconomic improvement. The pig population in India is only 9.06 million (20th Livestock Census, 2019) declined by 12.03% over the previous census. Our pig industry is facing various challenges, among which viral diseases pose major concern. It causes severe losses to pig industry by causing mortality, immune suppression which causes secondary bacterial infections, decreased feed conversion and reproductive problems and also by affecting carcass quality.

Postweaning multisystemic wasting syndrome (PMWS) is caused by porcine circovirus type 2 (PCV2) (Allan and Ellis, 2000; Saikumar and Das, 2019). It is a globally emerging clinical condition affecting nursing and growing pigs. PMWS was initially recognized in Canada in 1991. After that it has been detected worldwide in pig population. It is characterized by a low-grade fever, growth retardation, wasting, dyspnoea, anaemia, jaundice, diarrhoea and palpable inguinal lymphadenopathy with increased mortality in growing pigs between 4 to 15 weeks age group (Harding and Clark, 1997).

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Infection with PCV2 is crucial for development of PMWS (Kennedy *et al*. 2000). But PCV2 requires various cofactors for fully progress of PMWS. Recently, PCV2 was found persistently to be co-infected with other viruses, such as CSFV, porcine reproductive and respiratory syndrome virus (PRRSV) and PPV (Ouyang *et al*. 2019). CSF is one of the contagious and fatal trans-boundary diseases of swine and

caused by Pestivirus under Flaviviridae family. The most common pathomorphological lesions include tonsillitis, enlarged lymph nodes, haemorrhages on skin and different visceral organs like spleen, lung, intestine, kidney *etc*., button ulcers of the intestine, necrotic enteritis, non-purulent encephalitis *etc* (Blome *et al*. 2017). PPV is widely distributed virus among pig population and is the important cause of infectious infertility and SMEDI (stillbirth, mummification, embryonic death and infertility) syndrome in swine. PPV also aggravates PCV2 associated PMWS (Allan *et al*. 1999) due to increased production of TNF-α (Kim *et al*. 2006). Moreover, co-infection with multidrug resistant enteric *Escherichia coli* is of serious concern from veterinary clinical perspective and from zoonotic aspect. Thus, the aim of the present study was to describe the pathology of PMWS associated with CSFV, PPV and multidrug resistant enteric *Escherichia coli* infections in an organized swine farm, Rajasthan, India.

MATERIALS AND METHODS

Complete and detailed necropsy was conducted in a 2.5 months old male crossbred piglet from an organized swine farm, Rajasthan, India submitted to PM facility, division of pathology, ICAR-IVRI, Izatnagar in the year 2017 and proper recording of gross lesions of different organs was carried out. The farm was having history of 33% (200/300) of mortality among 2 to 6 months old piglets of various breeds such as crossbreds, large white Yorkshire and Landrace. The average litter size of that farm was 10 to 14 per dam with history of more than 45% stillbirth and mummification per dam. The affected animals showed various clinical signs like prominent spine bone, emaciation, respiratory distress, coughing, waving movement and mortality started after onset of diarrhoea. Heart blood and diarrhoeic intestinal contents were collected and stored separately at 4°C for bacteriological examination and antibiotic sensitivity test. Tissue samples were collected in sterile container and stored at -40°C for molecular diagnosis. Representative tissue samples from different organs were collected in 10% neutral buffered saline for histopathology.

Total DNA was extracted from pooled tissue samples for detection of PCV2 and PPV using QIAGEN DNeasy tissue kit as per the protocol mentioned in the kit and published primer pairs targeting to ORF2 gene and VP2 gene amplifying 263 bp and 226 bp products respectively were used. Amplifications were carried out in a thermal cycler (S1000TM Thermal Cycler, Bio-rad) according to PCR conditions described by (Larochelle *et al*. 1999 and Arnauld *et al*. 1998) respectively. For detection of CSFV, RNA was extracted from pooled tissues using TRIzolR reagents (Thermo Fischer Scientific, USA) as per standard procedures followed by complementary DNA synthesis using random primers and reverse transcriptase (RevertAid H Minus RT, Thermo scientific). Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using previously published primer pairs targeting E2 gene which amplify 272 bp product and amplifications were carried out according to RT-PCR conditions described by Tomer *et al*. 2020. The details of the primers used in the present study were listed in (Table 1). The PCR and RT-PCR amplicons were subjected to electrophoresis along with 100 bp plus molecular weight marker (Thermo scientific, USA) on a 1.5% agarose gel at 70V for 45 minutes. The gel was visualised under UV light and photographed in gel documentation system (GelDoc-ItTM, UVP).

For bacteriological examination, heart blood and faecal samples stored at 4°C were processed on brain heart infusion agar, blood agar, MacConkey Agar and Eosin Methylene Blue Agar using a standard loop and incubated at 37°C for 24-48 hours. Isolates were identified by colony morphology and gram staining (Bhattacharya, 2002). The antibiotic sensitivity test was carried out in Mueller Hinton agar using disc diffusion method as per standard procedures described by Bauer (Bauer *et al*, 1966).

For histopathology, tissue samples fixed in 10% neutral buffered saline were dehydrated, embedded in paraffin wax, sectioned at 4µm and stained with Haematoxylin and Eosin staining as per standard procedures (Bancroft and Gamble, 2008) which were microscopically examined and histopathological changes were recorded.

RESULTS AND DISCUSSION

Clinical signs and mortality

The organized farm located at Rasal, Rajasthan, India was having history of 33% (200/600) of mortality among 2 months to 6 months old piglets among various breeds such as crossbreds, large white Yorkshire and Landrace. Harding *et al*. 1998 reported postweaning mortality averaged 6.7% ±5.1% with maximum 18.2% in severely PMWS affected herd. The average litter size of that farm was 10 to 14 per dam with history of more than 45%-100% stillbirth and mummification per dam. As the farm was having high stillbirth and mummification, the piglets might be infected due to in utero transmission from naturally infected dam (Ladekjaer-Mikkelsen *et al*. 2001). The affected animals showed various clinical signs like prominent spine bone, emaciation, respiratory distress, coughing, waving movement and mortality started after onset of diarrhoea similar to previous reports of PMWS (Rossell *et al*. 1999).

Molecular diagnosis

The PCR and RT-PCR resulted in amplification of a 263 bp product from ORF2 gene of PCV2 genome, a 226 bp product from VP2 gene of PPV genome and a 272bp product from E2 gene of CSFV genome respectively (Fig 1). PPV DNA was detected in the lymphoid tissues of PCV2 infected pigs with PMWS by PCR suggesting dysfunction of immune system by PPV facilitates replication of PCV2 causing severe lesions of PMWS (Choi and Chae, 2000). Both PCV2 and PPV were reported to be associated with PMWS and reproductive failure in Indian crossbred pigs (Pegu *et al*. 2017; John *et al*. 2020). Deka *et al*. 2021 reported about

Table 1: Details of the primers used in the present study.

Primer names	Genestargeted	Sequences	Size of the amplicon	References
PCVL F	ORF2	5′-TAGGTTAGGGCTGTGGCCTT-3′	263 bp	Larochelle *et al.* 1999
PCVL R		5′-CCGCACCTTCGGATATACTG-3′		
PPV F	VP1	5′-CCAGCAGCTAACACAAGAAAAGGTTATCAC-3′	226 bp	Arnauld *et al.* 1998
PPV R		5′-GTCCATGTTGGTAATCCATTGTAAATC-3′		
M272E2 F	E2	5′- TCRWCAACCRAYGRGATAGGG-3′	272 bp	Tomar *et al.* 2020
M272E2 R		5′- CACAGYCCRAAYCCRAARTCATC-3′		

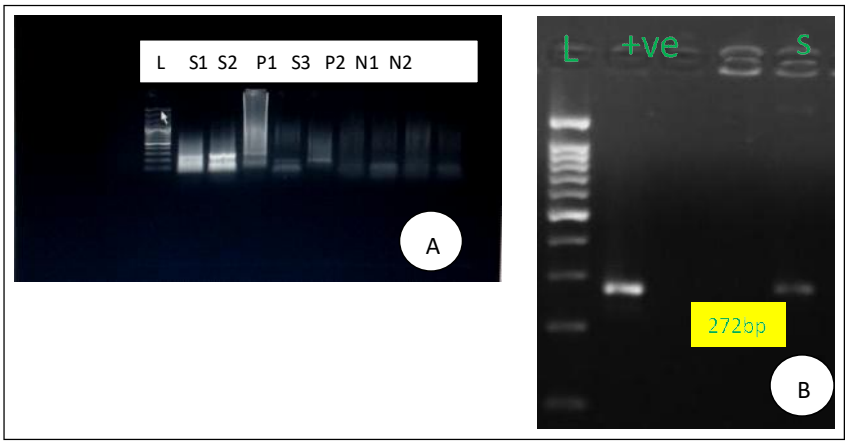

Fig 1: Ethidium bromide stained 1.5% agarose gel showing A. 268 bp and 226 bp amplicons of PCV2 (S1 and S2) and PPV(S3) respectively. B. 272bp amplicon of CSFV: Lane S: Sample, Lane L: Marker (100 bp), P1 and P2: Positive control, S1, S2 and S3: Sample, N1 and N2: Negative control.

1.75% seroprevalence of all CSFV, PCV2 and PPV among pigs of eight north eastern states and Punjab state of Northern India.

Bacteriological examination and antibiotic sensitivity test (ABST)

Bacteriological examination of heart blood revealed no bacterial growth but *Escherichia coli* was isolated from intestinal content. Isolates were characterized by pink colour colonies in MacConkey agar plate and greenish metallic sheen colonies in Eosin Methylene Blue Agar plate (Fig 2). The isolated E. coli was sensitive to morapenep, imipenem, doripenem, colostin, gentamicin and resistant to ciprofloxacin, ofloxacin, levofloxacin, enrofloxacin, cefotaxime, co-trimazole, kanamycin, nitrofurantoin, streptomycin, tetracycline, ampicillin, amoxyclov, carbenicillin, azoteconam. Lunha *et al.* 2020 also reported that *Escherichia coli* isolated from pig faeces in Thailand showed sensitive to meropenem and resistance to tetracycline (TET), trimethoprim/sulfamethoxazole (SXT), chloramphenicol (CHL) and gentamicin and the multidrug resistance was more common in pig isolates than human isolates and most common pattern was CHL-TET-SXT.

Gross lesions

Externally, the mucous membrane was pale with soiling of tail region. The inguinal, pre femoral and sub mandibular lymph nodes were very much enlarged and pale. Hydropericardium, gelatinization of pericardial fat, non-collapsed lung with patchy consolidations and catarrhal enteritis were observed. The bronchial and mesenteric lymph nodes were enlarged and congested. Liver was mildly enlarged and icteric. Kidneys were pale. Meningeal blood vessels of brain were congested (Fig 3). In Kerala, India; PMWS caused by PCV2 alone were reported in 5-12 weeks old piglets with presence of similar gross lesions (Sairam *et al.* 2019). Numerous button shaped ulcers were found distributed throughout the caecum and colon with severe necrosis of the mucosa of the caecum. The typical button shaped ulcer might be due to classical swine fever virus infection (Palanivel *et al.* 2012).

Histopathology

In lymph nodes, variable degrees of lymphoid depletion in the follicles and intense reticulo-endothelial (RE) cells proliferations were observed. In spleen, endothelial swelling and fibrinous thrombi in capillaries, severe lymphoid depletion in periarteriolar lymphoid sheaths and intense RE cell proliferations were noticed. In tonsil, lymphoid depletion was observed. Tracheal mucosa was highly congested. Lung showed severe interstitial pneumonia characterized by thickening of alveolar septa, infiltration of MNCs, compensatory emphysema and presence of oedematous fluid in some alveoli, degeneration and desquamation of bronchial and bronchiolar lining epithelium, depletion of BALT, endothelial cell swelling and fibrinous thrombi in the

capillaries. In heart, mild non suppurative myocarditis was noticed with degeneration of myocardiocytes, congestion and mild infiltration of MNCs. In liver, there was severe hepatitis characterized by both necrosis and apoptosis of hepatocytes, disorganization of hepatic cords, periportal MNCs infiltration and presence of multifocal histiocytic granuloma with intracytoplasmic botryoid inclusions in histocytes along with hepatocytes regeneration. Kidney revealed shrunken glomeruli, moderate to severe degenerative changes in kidney tubular epithelium, infiltration of MNCs in the interstitial space with focal areas of fibrous connective tissue proliferation. In small intestine,

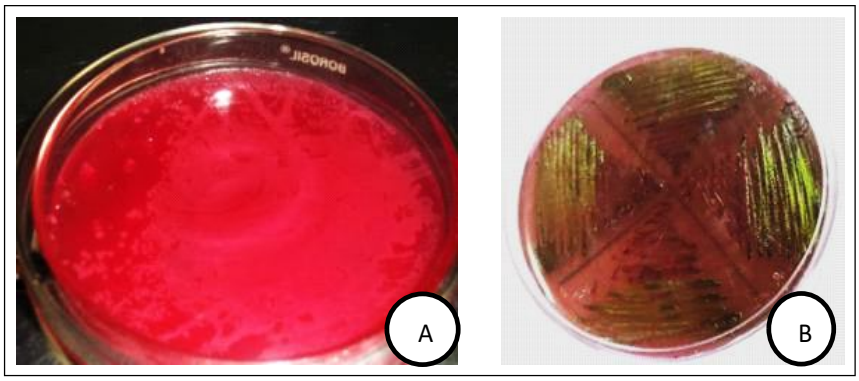

Fig 2: *E. coli* isolates. A. Characteristic pink colour colonies in MacConkey agar plate and B. Characteristic greenish metallic sheen colonies in Eosin Methylene Blue Agar plate.

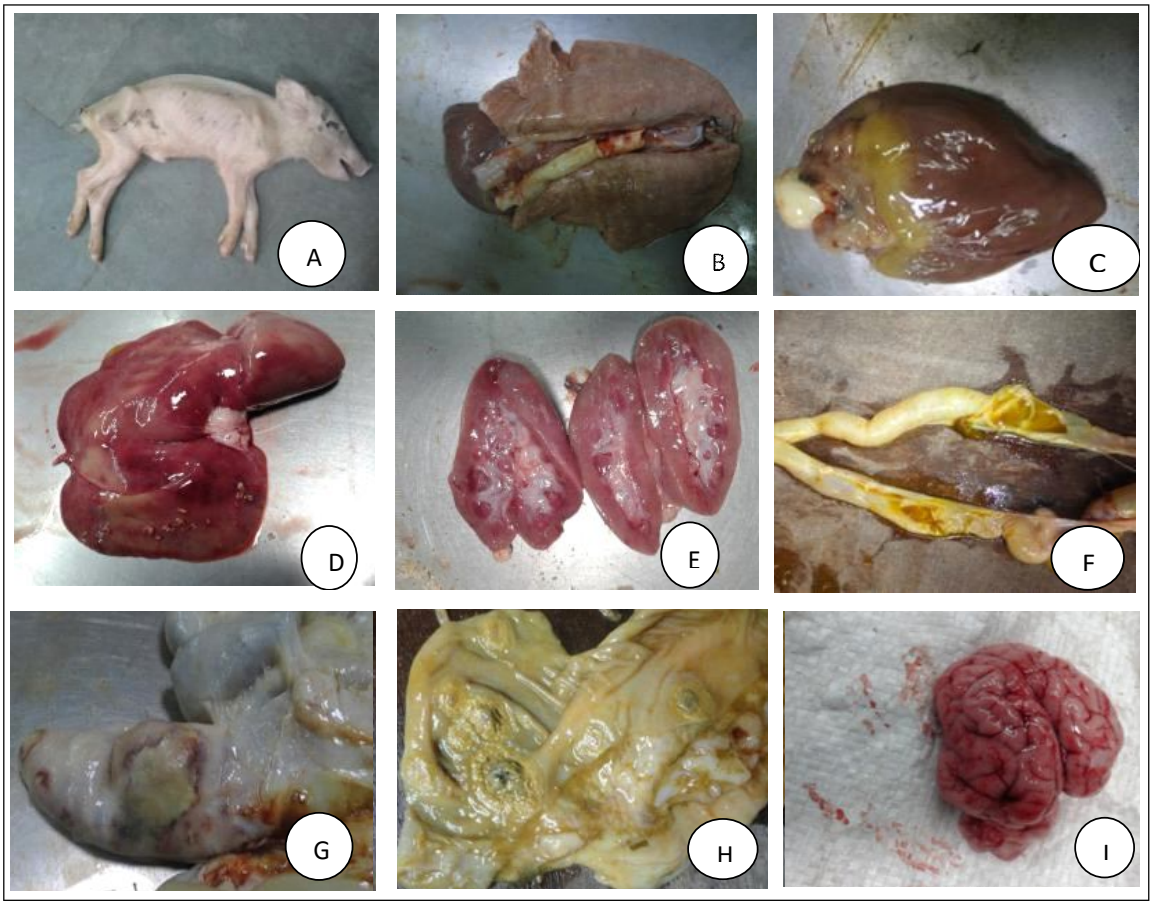

Fig 3: Gross lesions of PMWS affected piglet. A: Externally, the animal was very much emaciated with prominent rib bones and spinal column; B: Non collapsible lung with patchy consolidation; C: Gelatinization of pericardial fat; D: Mildly enlarged and icteric liver; E: Pale kidney; F: Bile-stained catarrhal content in the small intestine; G: Ulcerated serosal surface of caecum; H: Button shaped ulcers in caecum and colon; I: Congested meningeal blood vessels.

severe degeneration and necrosis of the mucosa with infiltration of inflammatory cells and depletion of lymphoid tissues were observed. In large intestine, presence of necrotic debris in the lumen, severe necrosis of the wall along with endothelial swelling and fibrinous thrombi in the capillaries were noticed. In cerebrum of brain, severe meningitis, perivascular cuffing, congested blood vessels, focal and diffuse gliosis and neuronal degeneration and necrosis were noticed (Fig 4).

Lymphoid depletion in mesenteric and inguinal lymphnodes along with hyperplasia of follicular dendritic cells were noticed in most of PMWS affected pigs (Roselle *et al*. 1999; Segales and Domingo, 2002). The prominent finding of PMWS was presence of sharply demarcated spherical intracytoplasmic inclusions in histocytic cells as observed by others (Sharma and Saikumar, 2014). Severe macrophage infiltration, granulomatous lesions and amphophilic inclusion bodies were reported in tissues of PPV and PCV2 co infected animals suggesting cofactors are important in the pathogenesis of PMWS (Kennedy *et al*. 2000). Interstitial pneumonia and interstitial nephritis are important histopathological findings in PMWS affected pigs (Wellenberg *et al*. 2004). In the present study, endothelial swelling and fibrinous thrombi were observed mostly in the capillaries and arterioles of spleen, lung and large intestine which might be due to PCV2 or CSFV infection. PCV2 infection induces procoagulation state in naturally infected pigs which induces vasculitis, endothelial cell activation and micro thrombi formation and haemorrhage (Marks *et al*. 2010). CSF virus causes extensive damage to the endothelial cells varying from swelling of endothelial cells to fibrinoid necrosis of capillaries and extensive formation of microthrombi in capillaries and arterioles (Cotran, 1994; Calderon *et al*. 1997). The histopathological lesions in brain

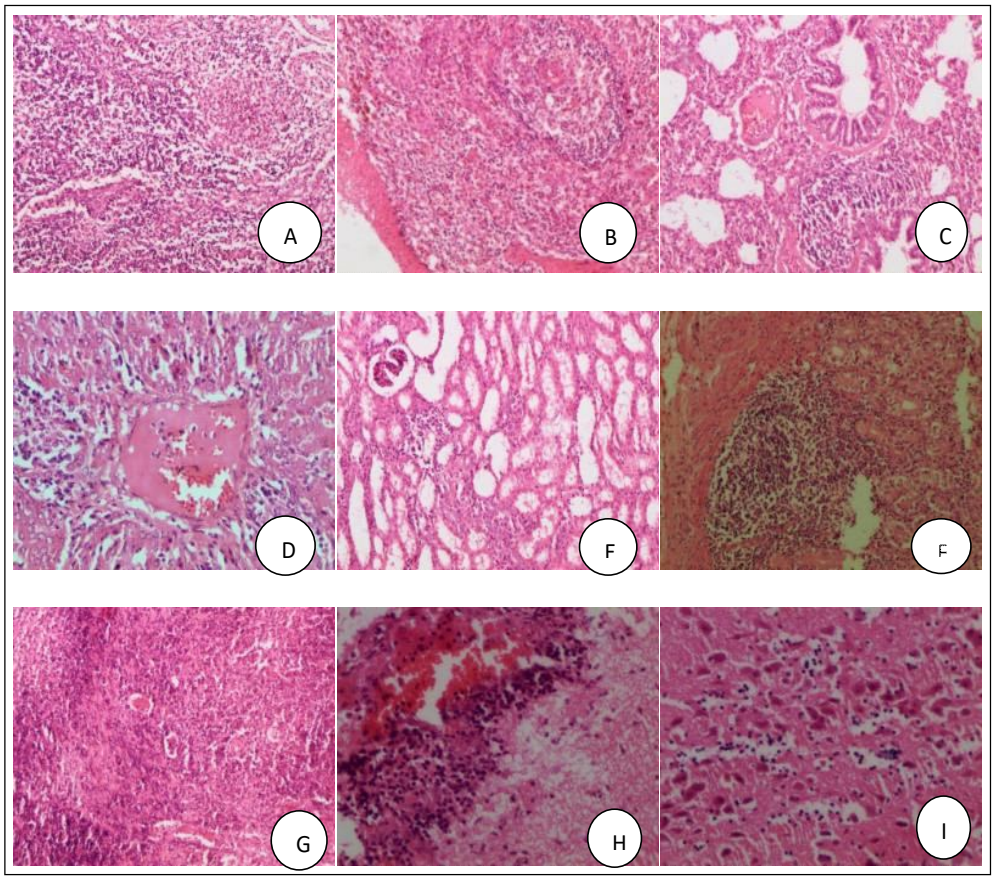

Fig 4: Microscopic lesions in PMWS affected piglets. A: Lymphoid depletion and RE cell proliferation in lymph nodes, H&E, X200; B: Presence of microthrombi, lymphoid depletion and RE cell proliferation in spleen, H&E, X200; C: Severe interstitial pneumonia, emphysema, degeneration and desquamation of bronchiolar epithelium, depletion of BALT and presence of fibrin thrombi in lung, H&E, X200; D: Congested central vein, necrosis and apoptosis of hepatocytes, MNCs infiltration and presence of multifocal histiocytic granuloma with intracytoplasmic botryoid inclusions in liver, H&E, X200; E: Shrunken glomeruli, degeneration of tubular epithelium, infiltration of MNCs in kidney, H&E, X200; F: Severe degeneration and necrosis of the mucosa with infiltration of inflammatory cells and depletion of lymphoid tissues of ileum, H&E, X200; G: Severe necrosis of the wall of large intestine along with fibrin thrombi in the capillaries, H&E, X200; H: PVC and congested blood vessels in the cerebrum, H&E, X400; I: Focal and diffuse gliosis and eosinophilic neurons in the cerebrum, H&E, X400.

mentioned here were similar to findings of another worker (Sharma and Saikumar, 2014).

CONCLUSION

Based on history, clinical signs, gross lesions, histopathology, bacteriological examination and molecular diagnosis, it was diagnosed as PMWS associated with CSFV, PPV and multidrug resistant *E. coli*. The synergistic action of these pathogens resulted in severe pathological lesions and mortality in post weaned piglets. In India, this is the first report on pathology of PMWS associated with all the three viruses and enteric colibacillosis.

Conflict of interest: None.

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