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Pathology and Molecular Diagnosis of Respiratory Disease Outbreak due to PCV-2 in a Pig Farm in Goa, India

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ABSTRACT

Background: Pig farming is an important source of income and nutrition for rural farmers in Goa. Pig production is threatened by many infectious diseases. The present study reports a respiratory disease outbreak in a total of 200 pigs including adults and grower pigs with 40% mortality in the herd of Large White Yorkshire and crossbreds in South Goa during the winter period in January 2022.
Methods: The farm was visited and recorded clinical signs and post mortem findings and samples were collected for bacterial isolation, PCR confirmation of bacterial and viral pathogens and histopathology.
Result: The affected pigs showed high fever, reduced feed intake, staggering gate, huddling, difficulty in breathing, cough and brown to greenish diarrhea followed by death within a span of 3 weeks. Major gross lesions were non-collapsed lungs with severe congestion and localized areas of consolidation, severe congestion of viscera and enlargement and presence of multifocal areas of necrosis in the liver. Histopathology of lungs revealed focal or diffuse bronchointerstitial pneumonia and the lymphoid organs showed lymphoid depletion. *Pasteruella multocida* could be isolated from heart blood and tissues from 3 cases and PCR of the tissue DNA confirmed the presence of *P. multocida* and Porcine Circo Virus-2 (PCV-2) infection. The study confirms the presence of PCV-2 infection in pig herds in Goa for the first time. The acute mortality can be attributed to the co-infection of the herd with PCV-2 and *P. multocida* and sudden change in weather patterns with prevailing severe cold conditions during time of outbreak. Sequencing and Phylogenetic analysis of the PCV-2 ORF-2gene showed the PCV-2 virus from Goa is more related to isolates from southern Indian states.

Key words: *Pasteurella multocida*, PCV-2, Phylogenetic analysis, Respiratory disease.

INTRODUCTION

Pig farming plays an important role in income generation as well as source of nutrition in rural Goa. Though there is a decrease in the pig population in Goa in the past 5 years, pig farming is still one of the profitable livestock enterprise. The productivity in pig farming is highly affected by the occurrence of disease outbreaks. Respiratory disease is regarded as one of the most common causes of mortality causing huge economic loss to the pork industry. Porcine respiratory disease complex (PRDC) a condition that occurs due to infection with multiple pathogens causes significant economic losses in the swine market by increased mortality, poor growth performance, cost of medications *etc*. (Maes *et al*., 1999; Harms *et al*., 2002; Calderon *et al*., 2020). Viruses like swine influenza virus (SIV), porcine circovirus type 2 (PCV-2) and porcine reproductive and respiratory syndrome virus (PRRSV) are important primary agents in PRDC and there are few bacterial pathogens that can also act as primary pathogens *viz. Actinobacillus pleuropneumoniae, Bordetella bronchyseptica* and *Mycoplasma hyopneumoniae*. Several other bacteria mainly *Glaesserella (Haemophilus) parasuis, Streptococcus suis, Salmonella choleraesuis* and *Trueperella pyogenes* can cause secondary infections resulting in exacerbation of respiratory clinical signs and lesions (Brockmeier *et al*., 2002). In India, the most commonly reported viral respiratory pathogens are Porcine Circo Virus-2 (PCV-2), Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Swine Influenza A viruses (SIV) and Classical Swine Fever Virus (CSFV) (Singh *et al*., 2022) and the major bacterial causes of respiratory diseases are *Pasteurella multocida* and *Streptococcus suis* (Rajkhowa *et al*., 2018).

PCV2 produces different manifestations like subclinical infection, post weaning multisystemic wasting syndrome (PMWS), pneumonia, enteritis, reproductive failure and dermatitis and nephropathy syndrome (Gillespie *et al*., 2009). It plays an important role in porcine respiratory disease complex (Harms *et al*., 2002). PCV-2 associated

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pneumonia affects pigs from 8 to 26 weeks old and the major clinical signs are decreased rate of growth, decreased feed efficiency, anorexia, fever, cough and dyspnoea (Opriessnig *et al*., 2007). The presence of prolonged and severe clinical respiratory disease, bronchointerstitial pneumonia with peribronchial and peribronchiolar fibrosis are indicative of PCV-2 associated PRDC (Kim *et al*., 2003). *Pasteurella multocida* is another widespread respiratory pathogen in pigs associated with which plays a significant role in porcine respiratory disease complex (PRDC). Pyrexia, mucopurulent nasal discharge and dyspnoea are reported in respiratory infection associated with swine pasteurellosis (Ghosh *et al*., 2011). The infection mainly manifests as broncho pneumonia with infiltration of mixed cell population and also systemically spread to a lesser extend to other organs (Pors *et al*., 2011; Kim *et al*., 2019). The present study reports a respiratory disease outbreak with high mortality in a pig herd in South Goa during winter period in January 2022.

MATERIALS AND METHODS

Sample collection

A pig farm located in the South Goa district recorded an outbreak of respiratory illness in January 2022. The affected farm was visited and clinical signs in diseased pigs were observed and post mortem examination was carried out on 3 dead pigs. The gross lesions were recorded and heart blood swabs, tissues including lymph nodes, lung, liver, spleen, kidney and heart from 3 dead carcasses were collected aseptically and blood samples from live pigs were collected and transported in ice to lab. Samples for RNA and DNA isolation were stored at -40°C and tissues were fixed in 10 per cent neutral buffered formalin for histopathology. The laboratory tests and analysis were carried out at ICAR-Central Coastal Agricultural Research Institute, Goa during 2022.

Pathogen identification

For bacterial isolation, the blood, spleen, lungs and liver samples were streaked on blood agar and bacterial colonies were identified and also confirmed by PCR using DNA isolated from bacterial colonies. DNA and RNA isolation were carried out from tissue and blood using Qiagen DNeasy Blood and tissue DNA kit and RNeasy Mini Kits followed by cDNA preparation using Himedia Hi-CDNA Synthesis kit and the DNA/cDNA samples were screened for suspected viral and bacterial pathogens viz. PCV-2 (Ellis *et al*., 1999), CSF (Pan *et al*., 2005) and PRRSV (Pegu *et al*., 2017), *Pasteurella multocida* (Townsend *et al.*, 1998), *Actinobacillus pleuropneumoniae* (Frey, 2003), *Haemophilus parasuis* (Oliveira *et al*., 2001), *Streptococcus suis* (Okwumabua *et al*., 2003). Primer details are given in Table 1.

Histopathology

The samples were processed for routine histopathological examination, stained using hematoxylin and eosin stain, a mounted with DPX mountant and examined under light microscope.

Phylogenetic analysis of PCV-2

From the swine tissue DNA, the PCV-2 *ORF-2* gene was partially amplified as previously described (Ellis *et al*., 1999) and sequenced. Comparison of the ORF-2 gene sequences of the Goan virus (present outbreak and a PCV-2 sequence from another outbreak in a farm at South Goa) with published sequences from other states of India and abroad, obtained from Gen Bank was carried out. The sequences were aligned in clustalW and subjected to Molecular Evolutionary Genetics analysis X (MEGA X) tool for evolutionary analysis. The phylogenetic studies were undertaken by the Maximum Likelihood method (MLT) with the Tamura-Nei model with the highest log likelihood (Tamura and Nei, 1993) to infer evolutionary relatedness. The pairwise nucleotide sequence distance matrix were calculated with Megalign program of DNASTAR software.

RESULTS AND DISCUSSION

Outbreak

A disease outbreak with 40 per cent mortality was observed in a swine farm in South Goa district having around 200 Large White Yorkshire pigs, of which 80% were of around 3-6 months of age and remaining were adults. Mortality of 62 grower pigs and 17 pregnant females were reported by the farmer. The disease was acute in onset and the affected animals showed sudden fever, staggering gate, huddling, difficulty in breathing, cough and brown to greenish diarrhea. The affected animals showed clinical signs for a short period of 1 to 3 days followed by death. The mortality occurred over a period of 3 weeks. Symptomatic treatment using antibiotics, dexamethasone, vitamin supplements and probiotics was given and only very few of the affected animals responded to treatment and recovered. The owner reported past occurrences of reproductive problems like abortion and still birth in the herd. As in the present outbreak, clinical signs like pyrexia, dullness, staggering gait, anorexia, serous nasal discharge and dyspnea were reported in swine Pasteurellosis (Tigga *et al*., 2015; Ghosh *et al*., 2011). Respiratory disease with fever and varying degrees of sneezing, coughing, nasal discharge and respiratory distress as well as reduced weight gain are important signs seen in PRDC16.

Diagnosis of pathogen

From blood samples and tissues *P. multocida* was isolated in blood agar. The colonies appeared small, glistening, mucoid, dewdrop-like and non-haemolytic and showed gram negative short rods which were suspected for *P. multocida* and were confirmed by PCR with amplification of 460 bp product. The PCR results showed that the blood, lung and liver tissue DNA of the 3 pigs were positive for *P. multocida*. Blood, liver, lung and spleen DNA of all the 3 pigs were positive for PCV-2 and PCR amplified 481bp product. The PCR results were negative for other bacterial and viral pathogens screened.

Gross lesions

Most of the dead pigs were in good body condition and only 2 showed patches of purple discoloration of the skin (Fig 1a). In all the examined pig carcasses, the lungs were non collapsed and had severe congestion and localized areas of consolidation and showed oozing of frothy fluid from the cut sites (Fig 1b). Other visceral organs the like spleen, liver, kidney and heart showed severe congestion. Liver was enlarged and showed multifocal areas of necrosis (Fig 1c). The lymph nodes appeared enlarged edematous and showed congestion. Congestion of mesenteric blood vessels and gastric mucosa was also noticed in few animals. Gross lesions including diffuse consolidation, emphysema and exudation of frothy fluid from cut site (Bhat *et al*., 2016) were described in swine Pasteurellosis. Lesions like generalized enlargement of lymph nodes, congestion of visceral organs like spleen, liver, kidney and stomach, focal or diffused areas of consolidation in lungs and liver enlargement and multifocal areas of necrosis observed in the present outbreak were similar to that reported earlier in PCV-2 associated disease cases (Sairam *et al*., 2019; Barman *et al*., 2018; Ghosh *et al*., 2011; Ellis *et al*., 1999).

Histopathology

Lungs showed congestion, edema and focal or diffuse broncho interstitial pneumonia. The columnar epithelium of bronchioles showed degenerative changes and the bronchiolar lumen was filled with edematous exudate and denuded epithelium (Fig 2a). Fibrous hyperplasia and extensive infiltration of mononuclear cells in the peribronchiolar area and hyperplasia of bronchial associated lymphoid tissue (BALT) was also observed (Fig 2b). In some areas, alveoli were coalesced, damaged and large bullae were seen with fibrinous proliferation in the interstitium. Most of the areas showed extensive infiltration of mononuclear cells in the interstitium and alveoli were collapsed or showed accumulation of edematous fluid in the alveoli (Fig 2b). In some areas alveoli were intact and the interstitium showed extensive thickening with accumulation of fibrin and mononuclear cells in the interstitial space (Fig 2c). These lesions were similar to that reported earlier in PCV-2 associated disease cases (Kim *et al*., 2003; Sharma and Saikumar 2008; Barman *et al*., 2018; Sairam *et al*., 2019; Hemalatha *et al*., 2020; Opriessnig and Langohr, 2013). Capillaries were engorged in most of the areas.

Lymph nodes showed lymphoid depletion with lesser number of lymphocytes in the germinal center. Cytoplasmic degenerative changes and necrosis of lymphoid cells were seen in the germinal centers (Fig 2d, 2e). In some samples, germinal centers and follicular structures were lost and clear demarcation between follicles and para-follicular areas were absent. In spleen similar changes were seen with severe lymphoid depletion and replacement with monocytes or macrophages with in the lymphoid follicles and extensive with hemorrhage is seen in the surrounding area. PALS also showed lymphoid depletion, replacement with macrophages and hemorrhage (Fig 2f). Plasma cells were also seen abundantly. Degenerative changes in the cytoplasm of lymphocytes and extensive lymphoid depletion were more prominent in the periphery below the capsule. The kidney showed congestion. The liver showed mild to severe capillary congestion between hepatic cords and accumulation of edematous fluid in parenchyma. Mild focal

Fig 1a: Dead carcass showing purple discoloration of skin.

Fig 1b: Lungs showing focalized consolidation, presence of frothy exudate at the cut site.

Fig 1c: Liver showing multiple necrotic foci in the parenchyma.

mononuclear cell infiltration was observed around portal canal in some samples. Similarly, diffuse lymphoid depletion with reticulum cell hyperplasia in spleen and mild multifocal coagulative necrosis with lymphoplasmacytic infiltration around the portal areas in liver were earlier reported in PCV-2 infections (Rosell *et al*., 1999; Sharma and Saikumar, 2008; Hemalatha *et al*., 2020).

Porcine respiratory disease complex (PDRC) is one of the most common health problems in pig production systems mainly affecting finishing pigs from 14 to 22 weeks of age which can cause economic losses due to deaths, low weight gain, treatment cost and condemnation of carcasses. Porcine respiratory disease complex (PRDC) is a multifactorial disease and morbidity ranges from 10% to 40% and mortality from 2% to 20% (Kim *et al*., 2003; Harms *et al*., 2002). Environment, type of production system and management practices are significant predisposing factors to PRDC. In order to meet the requirement for meat, live pigs are brought to the Goa state for slaughter from neighboring states, which is an important factor in the spread of new diseases. The present study shows that the mortality occurred due to the mixed infection with PCV-2 and *P. multocida*. PCV-2 is a commonly reported as a cause of multisystemic disease in pigs, but it is also often associated with pulmonary lesions (Opriessnig and Langohr, 2013). As the present case was seen as an acute outbreak of fever and respiratory disease for a short duration of 3 to 7 days and the animals were healthy with good body weight prior to the start of clinical signs it shows that it is an outbreak of PCV-2 associated PRDC and not postweaning multisystemic wasting syndrome (PMWS). Pneumonia associated with porcine circovirus type 2 (PCV2) is frequently reported in respiratory disease cases in growing pigs. Even though Pasteurellosis was confirmed by isolation and PCR, the pulmonary lesions characteristic of pneumonic pasteurellosis like suppurative bronchopneumonia, abscessation, focal area of necrosis, polymorphonuclear cell infiltration, pleuritis, *etc*. were not noticed in the present outbreak. The interactions between the infecting pathogens play an important role in developing PRDC. The histopathology of lungs in present case showed lesions characteristic of PCV-2 associated PRDC. *P. multocida* is generally considered to be an opportunistic invader that is rapidly cleared from the lungs of normal pigs (Rosell *et al*., 1999). In pigs, it is associated with progressive atrophic rhinitis (Davies *et al*., 2003) and is frequently isolated from PRDC as co-infections with PCV-2 (Maes *et al*., 1999). PCV2 is an important primary pathogen causing PRDC, which affects lymphoid tissue, causing lymphocyte depletion and immunosuppression or immune dysfunction in pigs (Opriessnig and Langohr, 2013). In the

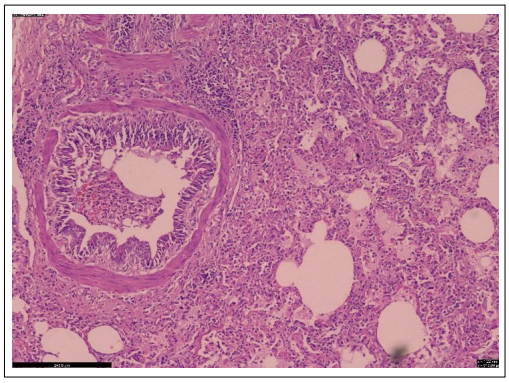

Fig 2a: Lung section, columnar epithelium of bronchioles showing degenerative changes and the bronchiolar lumen filled with edematous exudate and denuded epithelium (H&E staining 10×).

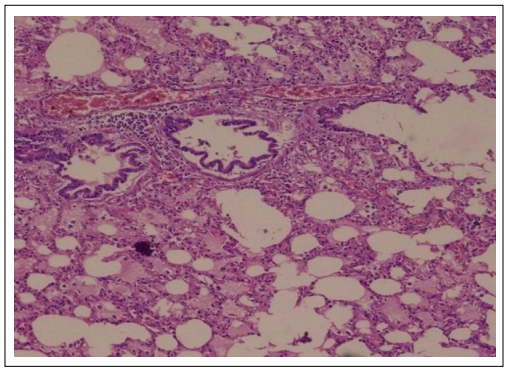

Fig 2b: Severe congestion of capillaries, coalesced alveoli forming bullaes and accumulation of edematous fluid in the alveoli. (H&E staining 4×).

Table 1: Oligonucleotide pairs used for PCR screening.

Streptococcus suis	JP4 F GCAGCGTATTCTGTCAAACG JP5 R CCATGGACAGATAAAGATGG	Okwumabua *et al*., 2003
Pasteurella multocida	KMT1T7F ATCCGCTATTTACCCAGTGG KMT1SP6R GCTGTAAACGAACTCGCCAC	Townsend *et al*., 1998
Haemophilus parasuis	HPS F GTGATGAGGAAGGGTGGTGT HPS R GGCTTCGTCACCCTCTGT	Oliveira *et al*., 2001
Actinobacillus pleuropneumoniae	AP F1 TGGCACTGACGGTGATGAT AP R1 GGCCATCGACTCAACCAT	Frey, 2003
PCV-2	PCV2 1443 F CGGATATTGTAGTCCTGGTCG PCV2 150 RACTGTCAAGGCTACCACAGTCA	Ellis *et al*., 1999
CSF	HCF ACYCTGACYGGCTGCAAGAAAGG HCR CCCCCCATYTCATGRAGAATCTT	Pan *et al*., 2005

present outbreak, *P. multocida* could be a secondary infection due to PCV-2 infection.

Phylogenetic analysis of PCV-2 virus

The PCV2 *ORF2* gene was amplified from tissue samples of two pig carcasses from the present outbreak and a pig sample from another farm at South Goa and were sequenced at Eurofins Genomics of India Pvt. Ltd, Bangalore and were labelled as Ind Goa P2 and Ind Goa P3. Phylogenetic analysis was carried out along with published sequences of 21 isolates from different states of India isolated between 2007 to 2021 and 7 isolates from other countries isolated between 2004 to 2018 using MEGA × (Fig 3) which revealed that the 31 PCV-2 sequences were broadly grouped into five distinct clusters/clade. The Goan PCV-2 sequences were grouped closely with isolates from North India (GU808525) and Kerala (MW627194) and other few isolates from India, China and Korea were also present in cluster-I. The pairwise distance matrix for nucleotide showed that the Goan isolates, Ind Goa P2 and Ind Goa P3 from the present outbreak were 100% identical and shared 98.9% identity with the Ind Goa 231. Also the Ind Goa P2 and P3 shared highest nucleotide identity (98.9%) with isolate from south Indian states of Andhra Pradesh (MW790263) and Kerala (MW268739). The Goan PCV-2 Virus Ind Goa 231 had 98.9% identity with recent isolates MZ254668 from Assam isolated in 2020 and MZ254670 (Arunachal Pradesh, 2020). This isolate also showed 98.7% identity with those from China (MN170528), USA (MW051676) and South Korea (MT376345). Past reports have shown that isolates from Uttar Pradesh shared a nucleotide identity of 94.7-98.1% with PCV2 isolates from China, United Kingdom and Germany based on complete genomic sequences (Anoopraj *et al*., 2015) and isolates from South Indian states shared more than 99% sequence homology to PCV2 sequences from Asian countries such as Taiwan, South Korea, China, Thailand and Vietnam based on *ORF2* gene sequence (Parthiban *et al*., 2022).

In Indian pigs, PCV2 was detected in 2006 from North India (Kumar *et al*., 2006). The presence of the virus has been studied by molecular detection and seroprevalence in swine herds of northern India (Anoopraj *et al*., 2015; Deka *et al*., 2021), North Eastern states (Pegu *et al*., 2017; Barman *et al*., 2018; Mukherjee *et al*., 2018; Bhattacharjee *et al*., 2021) and southern states (Karuppannan *et al*., 2016; Keerthana *et al*., 2019; Sairam *et al*., 2019; Hemalatha *et al*., 2020; Parthiban *et al*., 2022). Even though PCV-2 was not diagnosed earlier in Goa, the incidence of reproductive problems like abortion and still birth was reported in some

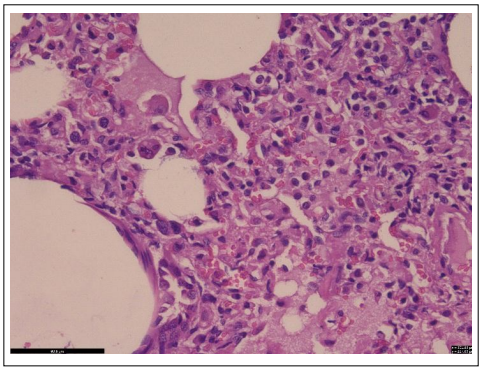

Fig 2c: extensively thickened interstitium due to mononuclear infiltration, accumulation of edematous fluid and fibrin (H&E staining 40×).

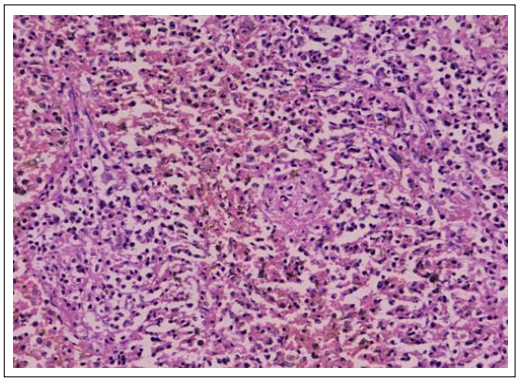

Fig 2d: Spleen, severe lymphoid depletion around germinal centre and replacement of lymphocytes around germinal centre by monocytes and macrophages and haemorrhage in the follicle (H&E staining 20×),

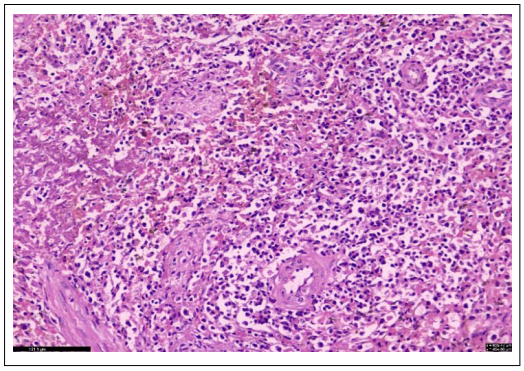

Fig 2e: Spleen highly necrosed germinal centre with severe lymphoid depletion and haemorrhage in the follicle (H&E staining 20×).

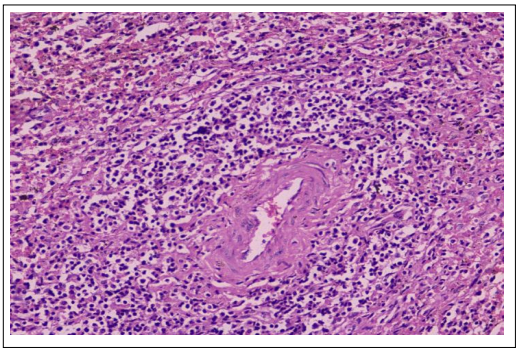

Fig 2f: Spleen, severe lymphoid depletion and replacement with large monocytes/macrophages in the Pals (H&E staining 20×).

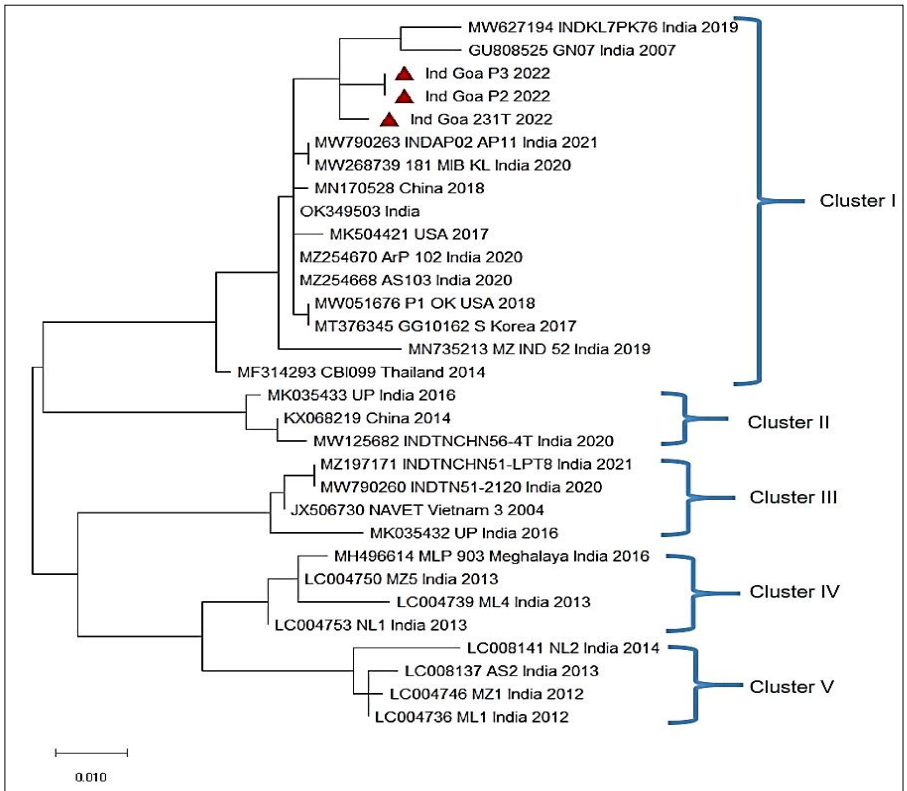

Fig 3: Phylogenetic analysis of PCV2 based on partial ORF2 sequences obtained from present study (Indicated by coloredtriangle) 28 PCV2 published sequences from Gen Bank.

farms and the farm where the present outbreak occurred had a history of reproductive problems. The present study confirms the presence of PCV-2 in a pig farm in Goa for the first time. Movement of people, transport of pigs, pig products *etc*. could be responsible for the spread of the virus to the new area. Hence identification of circulating field strains and the study of the phylogenetic relationships are essential to monitor the distribution and genetic diversity of PCV2 in the swine farms in the region. The phylogenetic analysis shows the close genetic relatedness of Goan PCV-2 with an isolate from North India and two isolates from southern states. The results of the phylogenetic study show that strains found in Goa are closely related to isolates from Northern India and South Indian states of Kerala and Andhra Pradesh. Goa state borders with Karnataka and Maharashtra states and no sequence was available of isolates from these states.

CONCLUSION

In conclusion the study confirms the presence of PCV-2 infection in pig herds in Goa for the first time. The acute mortality can be attributed to the co-infection of the herd with PCV-2 and *P. Multocida* and sudden change in weather patterns with prevailing severe cold conditions during time of outbreak. Phylogenetically the PCV-2 virus from Goa is more related to isolates from southern Indian states. As the virus can cause disease in all age groups and can lead to immunosuppression and predispose the herds to multiple other pathogens prevalence of this virus in Goa can cause huge economic loss to pig farmers hence vaccination against PCV-2 in the Goa state seems necessary.

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Conflict of interest

The authors declare that they have no conflict of interest.

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