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Molecular characterization and phylogenetic analysis of African swine fever virus from a pig farm in India

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Abstract

African swine fever (ASF) is considered as one of the most threatening diseases for the pig farming industry all over the world. Due to the lack of an effective vaccine, organized farms and backyard rearing must strictly enforce control measures in order to combat the disease. The present report describes the ASF epidemic in a piggery in Uttar Pradesh state, India. The pathological samples were collected from the affected pigs and processed for histopathological and molecular studies. Gross lesions comprised of cyanosis of ear pinna, multi-focal hemorrhagic spots on ventral abdomen and inner aspect of thigh, highly congested mesenteric lymph nodes with marbling, marked congestion, hemorrhages and splenomegaly, interstitial pneumonia, and multi-focal endocardial hemorrhages on papillary muscles and wall of ventricle in heart. Histopathological investigation revealed marked congestion and hemorrhages of mesenteric lymph node, liver and spleen. Depletion of lymphocytes from the splenic white pulp was visible in the splenic parenchyma. The virus was confirmed by polymerase chain reaction and phylogenetic analysis revealed a distinct clustering of the Uttar Pradesh virus isolates from Vietnam with other Ib group isolates, indicating a close genetic relationship between these samples. Additionally, the mutant Chinese virus isolate showed clear genetic differences with the Vietnamese Ib group, confirming its suitability as an out-group for comparison. The study represents the first report of ASF outbreak in North India, establishing the phylogenetic relationship between ASF virus circulating in the study area and other regions.

Introduction

The African swine fever (ASF), a fatal disease of pigs and wild boars, is extremely contagious, with high mortality and has become a severe economic threat to the pig farming industry with worldwide implications.[1] The disease is caused by ASF virus (ASFV), belonging to the *Asfivirus*genus of *Asfarviridae* family.[2] According to the World Organization for Animal Health, the disease is notifiable. The disease was initially identified in 1921 in Africa and became endemic. These days, the disease occurs in many countries affecting pig population.[3,4] The recent expansion of ASF to China, Mongolia, and Southeast Asia has highlighted this issue more than ever.[5] Not only it is harming the livelihood of pig farmers in rural areas, but also inflicting enormous losses to the swine industry. The ASF is clinically characterized by high fever, anorexia, respiratory problems and sudden death, while the most common pathological lesions are petechial hemorrhages in kidney, splenomegaly, enlarged liver and hemorrhagic lymph nodes.[6] Depletion of lymphocytes in the lymph nodes and spleen is the remarkable feature of ASFV infection, when viewed under a microscope.[7,8] Backyard farming is considered as a prone to disease introduction due to the poor management practices. In India, there are 9 million pigs and the majority (45.00%) of these pigs is reared in the northeastern states of the country, where pork is the primary food source. In May 2021, eleven outbreaks of ASF were reported in northeastern states (Assam and Arunachal Pradesh), where 3701 pigs perished from ASF, which was the first occurrence of the disease in the country. Furthermore, many northeastern

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states share porous borders with Bangladesh, Myanmar, Tibet, and China.[9] This case report throws a light into the clinical signs, post-mortem lesions, histopathological findings, molecular characterization and phylogenetic analysis of ASFV from an outbreak at a pig farm located in Uttar Pradesh state, India.

Materials and Methods

Study area and management practice. The affected pig farm was located in the Ghaziabad district of Uttar Pradesh state, India. The landscape of the village is dominated by agricultural fields on the plains. In the affected farm, the pigs were housed in closed housing, where the pens were approximately 6 - 7 m^2. Water was supplied by hoses in each pen coming from the underground water source. Pigs were fed with hostel waste and sometimes pre-mixed compound feed produced from feed manufacturers. According to the animal owner, the laborer along with his family was mainly taking care of the animals. Stable amenities were situated on the brick-walled, gated property. All pigs were purchased 1-2 years back, when the farmer decided to start pig farming.

Timeline of the disease event. In the mid-February, 2023, one fattening pig was found dead after it had been medically treated by the local veterinary staff due to the non-specific clinical signs (inappetence, vomiting and lethargy) being shown the days before. After 10-15 days, two fattening pigs died, after being treated medically (antibiotics and non-steroidal anti-inflammatory drugs) because of showing similar unspecific clinical signs. Few days later, other few growing pigs died under comparable circumstances and were buried by the farmer together with the carcass of the pigs. On 24 March, the animal owner contacted the College of Veterinary and Animal Sciences, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, India, for the post-mortem examination to know the cause of the disease. Necropsy was performed and tissues, such as liver, spleen, lung, heart, intestine, kidney and lymph node of the female pig were collected in strict biosecurity measures for further laboratory examinations.

Pathomorphological changes. The female pig carcass had severe pathological changes that were noted. The tissue samples were cut into the thin slices of about 1.00 cm and collected in 10.00% neutral buffered formalin solution for histopathological examinations. The samples were treated using the standard dehydration and cleaning procedure using alcohol and xylene. Paraffin embedding was carried out using paraffin wax, and sections were cut using a rotary microtome (Leica Microsystems, Wetzlar, Germany) at a thickness of 4 - 5 µm. The sections were stained with standard Hematoxylin and Eosin stain, mounted with dibutylphthalate poly-styrene xylene (HiMedia, Thane, India) and seen under a light microscope.[10]

Detection of viral nucleic acid. For making the homogenized 10.00% (w/v) solution, the tissue samples were manually homogenized using a sterile pestle mortar and phosphate buffer saline (pH: 7.40). The DNeasy Blood and Tissue Kits from Qiagen, Hilden, Germany, were used to extract the entire genome's DNA. The NanoBio 3.0 Spectrophotometer (Analytica, Mumbai, India) was used to evaluate the purity of the isolated genomic DNA, which was then kept at – 20.00 ˚C for later processing. Polymerase chain reaction (PCR) was performed as *per* the methodology recommended by World Organization for Animal Health.[11] A 257 base pair (bp) fragment of the ASFV was amplified using the PPA-1/2 primer set Forward (5′-AGTTATGGGAAACCCGACCC-3′) and Reverse (5′-CCCTGAATCGGAGCATCCT-3′) primers. The PCR reaction was performed using PCR Master Mix (Takara, Kusatsu, Japan) in an automatic DNA thermocycler (Takara). A 25.00 µL reaction volume was performed with 200 ng of purified genomic DNA. The PCR conditions consisted of a 1st denaturation step of 10 min at 95.00 ˚C, followed by a 2nd step of 40 cycles of denaturation (15 sec at 95.00 ˚C), primer annealing (30 sec at 62.00 ˚C), and extension (30 sec at 72.00 ˚C) along with a final extension step of 7 min at 72.00 ˚C. A total of 10.00 µL amplified PCR product was used in the 1.00% agarose gel electrophoresis containing 10.00 µL *per* mL ethidium bromide. The genomic DNA of the virus was reconfirmed using different primers to amplify 478 base pairs (bp) fragment of ASFV gene (partial *B646L* gene) using Forward (5′-GGCACAAGTTCGGACTGT-3′) and Reverse (5′-GTACTGTAACGCAGCACAG-3′) primers.[12] The tissue samples were also sent to ICAR-National Institute of High Security Animal Disease, Bhopal, India, for further confirmation.

Nucleotide sequencing and analysis. In order to confirm the identity of the viruses, the amplified product of 478bp size was excised and purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, USA). The 100ng of the purified amplicons were subjected to direct Sanger sequencing using both forward and reverse primers at Centyle Biotech, New Delhi, India. Thereafter, the quality of the obtained sequences was checked, and the ends of the sequences were trimmed using MEGA Software (version X; Biodesign Institute, Tempe, USA). The nucleotide sequences of the *p72* gene were subjected to multiple sequence alignment using CLUSTAL W, together with other representative nucleotide sequences of the ASFVs available on the National Center for Biotechnology Information. Phylogenetic analysis was performed using MEGA. The GenBank database received the Meerut ASFVs' *p72* sequences, being accessible under the accession numbers of OR062230 and OR062231.

Results

Gross pathology. The carcass was in good condition and post-mortem examination revealed several gross alterations in different organs of the affected animal (Fig. 1). Gross lesions consisted of mild erythema and cyanosis of ear pinna, and blotching and multi-focal hemorrhagic spots on ventral abdomen and inner aspect of thigh. The abdominal cavity was filled with serosanguineous fluid. Although gastrointestinal tract was apparently normal, the mesenteric lymph nodes were highly congested and hemorrhagic, displaying marbling. There were marked congestion, hemorrhages and splenomegaly. Lymph nodes showed varying degrees of enlargement, congestion, hemorrhages and marbling. Lungs were edematous and non-collapsing because of interstitial pneumonia. Trachea revealed presence of froth, suggesting alveolar edema or struggling before death. Multi-focal endocardial hemorrhages were also recorded on papillary muscles, and wall of the ventricle in the heart. Grossly, the liver was mildly swollen, while the kidneys were normal. The gross alterations observed during post-mortem examination indicated the possibility of ASF.

Histopathological changes. Histopathological investigation revealed several characteristic microscopic alterations in various tissues of ASFV-infected pig (Fig. 2). The intestinal mucosa, particularly of ileum, showed disruption of villi and muscularis mucosae at places, and infiltration of eosinophils and mononuclear cells in mucosa and sub-mucosa. Though the liver was grossly normal, microscopically there was dilatation of sinusoids and hemorrhage was displayed at places. The spleen showed marked congestion and hemorrhages and the splenic parenchyma showed depletion of lymphocytes from the splenic white pulp. There were congestion of the capillaries and hemorrhages in lymph nodes. The depletion of the lymphoid follicles was prominent. In some of the areas of liver, infiltration of mononuclear cells in peri-portal areas was seen. The lung revealed marked interstitial pneumonia, thickening of inter-alveolar septa due to the marked increase in edematous fluid, and infiltration of mononuclear inflammatory cells.

Molecular characterization. The amplicons of 257 bp and partial *B646L* gene (478 bp) were amplified from the genomic DNA isolated from the tissue samples of the affected pig using PCR (Fig. 3). The tissues were also found positive for ASF at ICAR-National Institute of High Security Animal Disease, Bhopal, India. In the present study, the genetic analysis of Meerut virus sequences (OR062230 and OR062231) from Vietnam virus sequences (ME828879, MW828875, MW828876, and MW825002) was conducted by specifically clustering them with Vietnamese isolates in Ib group, and used a mutant Chinese virus isolate (OL804293) as an out-group for comparison of the phylogenetic tree (Fig. 4) and identity matrix (Fig. 5).

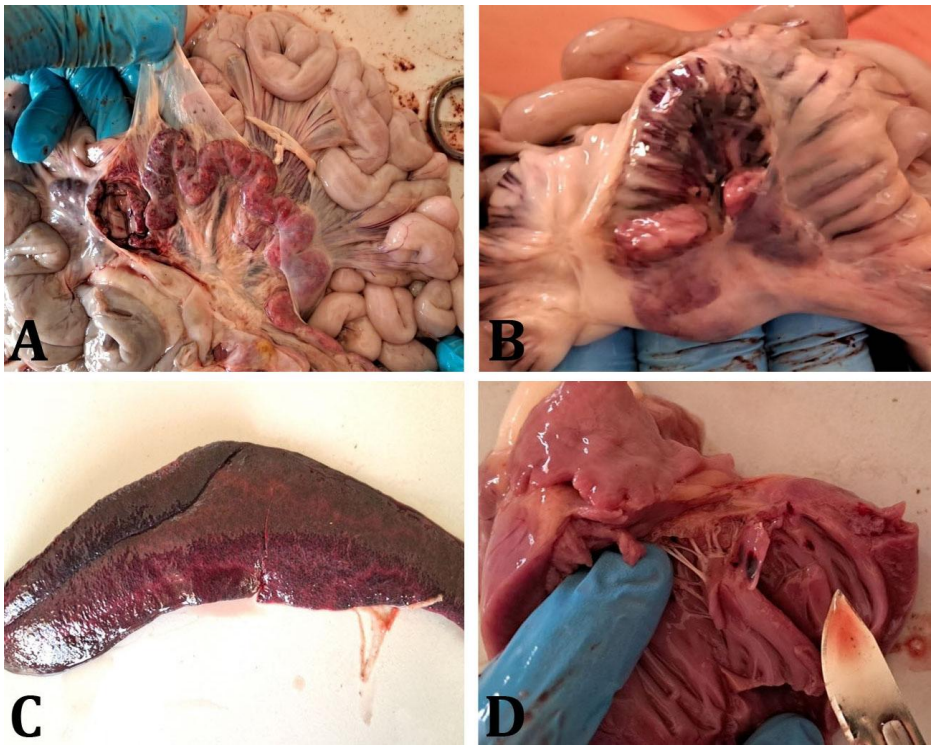

Fig. 1. Gross alterations in African swine fever virus-infected tissues. **A)** Mesenteric lymph nodes showing marked enlargement, congestion and hemorrhage. **B)** Cut surface of mesenteric lymph node showing hemorrhage and marbling. **C)** Spleen showing marked congestion, hemorrhage and spleenomegaly. **D)** Heart showing endocardial hemorrhage on papillary muscles and wall of ventricle.

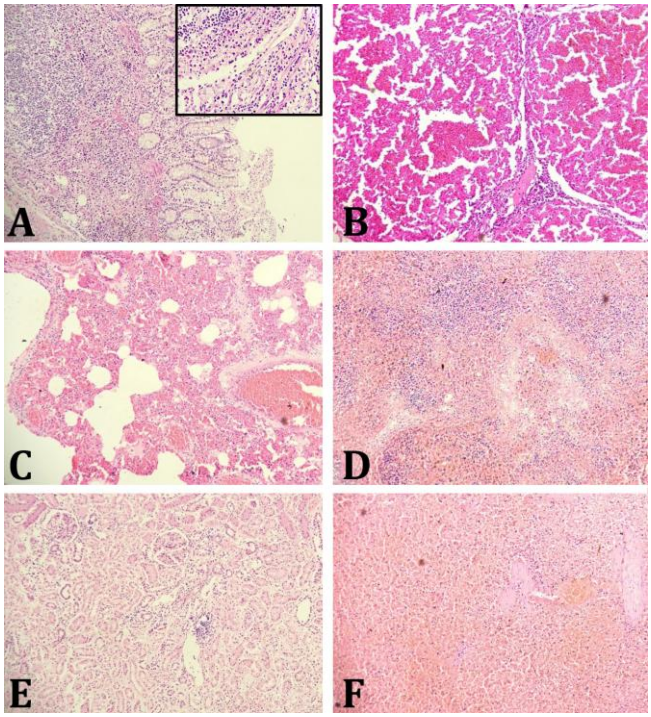

Fig. 2. Histopathological examination of African swine fever virus-infected tissues using Hematoxylin and Eosin staining. **A)** The microscopic section of ileum showing goblet cell hyperplasia, disruption of muscularis mucosae, disintegration and atrophy of villus epithelium, and infiltration of inflammatory cells in tunica mucosa (100×). Inset: Ileum showing marked infiltration of eosinophils in both the mucosa and sub-mucosa (400×). **B)** Liver showing marked hemorrhage of hepatic lobule, hemosiderosis, sinusoidal dilatation and peri-portal infiltration of mononuclear inflammatory cells in hepatic parenchyma (100×). **C)** Lung showing interstitial pneumonia, exudation and infiltration of inflammatory cells in inter-alveolar septa, congestion and edema (100×). **D)** Lymph node showing marked hemorrhage, congestion, hemosiderosis, apoptotic bodies and depletion of lymphoid cell population (100×). **E)** Kidney showing mild tubular degeneration and focal area of mononuclear cell infiltration (100×). **F)** Spleen showing marked congestion, hemorrhage, apoptotic bodies and hemosiderosis (100×).

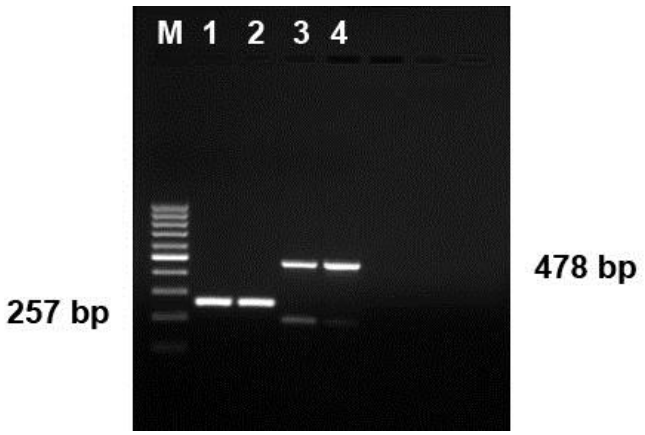

Fig. 3. Amplification of African swine fever virus gene. Lane M: 100 bp plus DNA ladder; Lanes 1-4: Tissue sample.

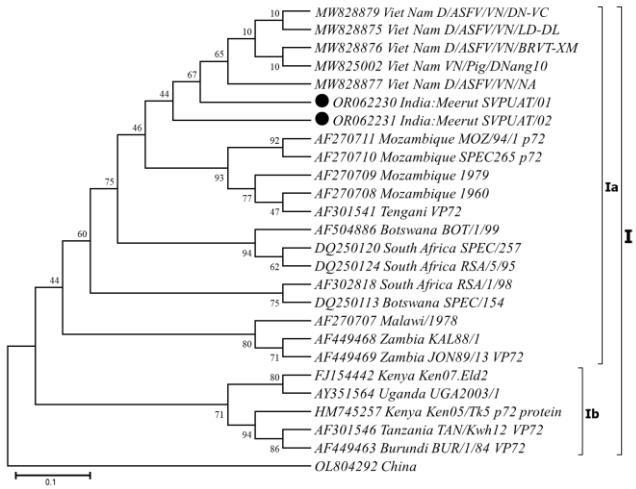

Fig. 4. Phylogenetic tree of the Meerut isolates of African swine fever virus.

Discussion

Clinical manifestations of AFSV infection can range from chronic, sub-clinical, or low-level sickness to hemorrhagic fever and sudden mortality. Similar pathological changes are seen in domestic pigs with ASFV infection. This paper reports the identification of ASFV in farm pigs from Uttar Pradesh state, India, its post-mortem lesions and histopathological findings. The gross pathological examination of pig carcass in the present study is similar to the previous reports.[8,13,14] Pig with the ASFV infection has been observed to have similar types of cyanosis in skin of various body regions, and petechial hemorrhages in intestines.[7,8] Contrary to the present findings, the hemorrhages on the surface of the kidney were reported in extensive research on the pathological alterations in ASFV-infected kidneys.[15-17] The prominent or marked splenomegaly,[18,19] cyanosis in skin of various body regions,[20] and different degrees of hemorrhages in the gastrointestinal tract[7] and lymph nodes[21] in the present study indicated the possibility of ASF. One of the noticeable features of ASFV infection is immunosuppression due to the lymphoid depletion induced by lymphocytic apoptosis.[7,8] Similar to the previous studies, a decrease in lymphocytes was observed in various lymphoid organs, such as spleen and lymph nodes.[19,20,22] Similar to previous reports, mononuclear cells, cell debris, and fibrin deposits were found in the splenic red pulp.[8,21] The other microscopic changes in various organs, such as lung, liver, heart and intestine were comparable to those seen in pigs infected with ASFV in the past.[7,8,18,19,21,23] Mononuclear cells depletion and apoptosis in lymphoid organs of present case report clearly suggest the viral tropism towards these cells. The present study is in line with previous study suggesting the mononuclear phagocytic cells as a

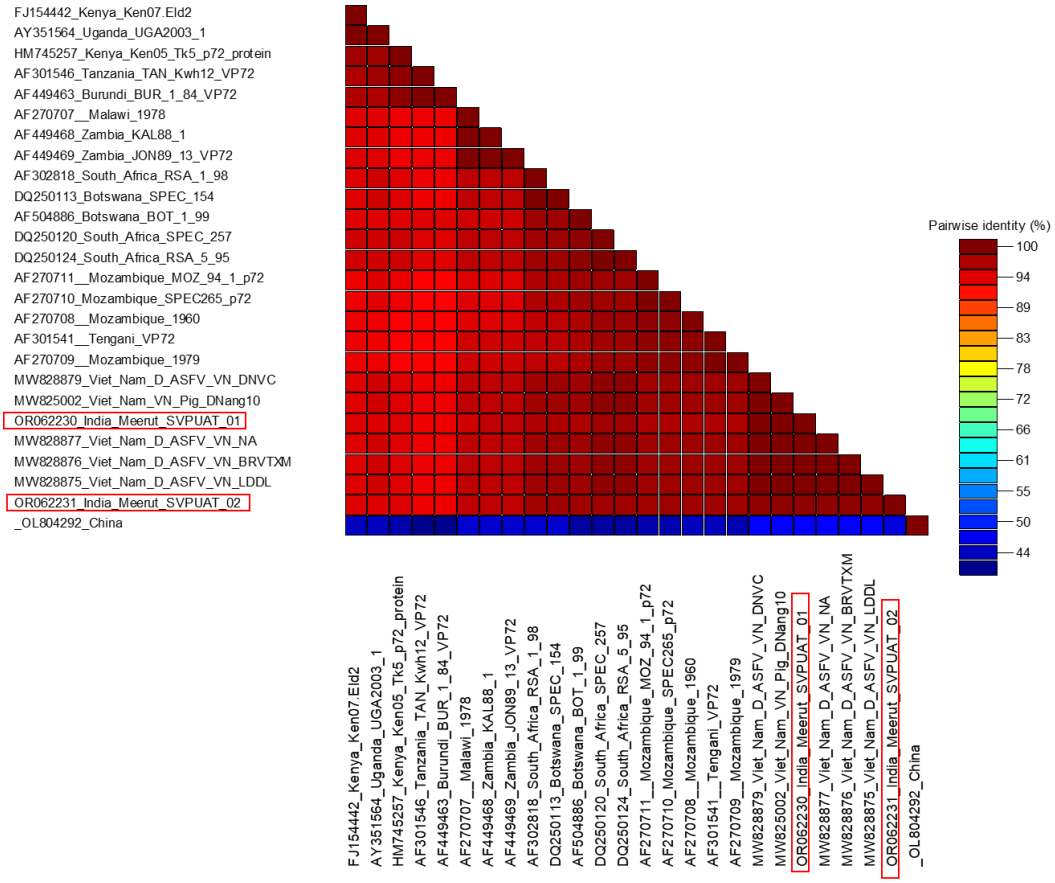

Fig. 5. Identity matrix of Meerut African swine fever virus with previously reported African swine fever virus sequences.

major site of virus replication.[24] One of the important findings in the present study is hemosiderosis of lymph node and spleen which might be due to adsorption of viral particles on erythrocytes, making them recognizable by the sentinel cells of body phagocytizing these erythrocytes and store iron as hemosiderin after digestion appearing as a brown deposit (hemosiderosis).[25]

The time between ASFV may have existed on the farm and the disease suspected is called high risk period (HRP), being an important criterion for effective tracing back of the possible disease spread.[26] For this, the laboratory results and mortality statistics were taken into account. The first pig succumbed from an ASF infection in mid-February. The most likely timing of disease introduction was the beginning of February 2023, with an expected survival time of roughly 10 days after infection. Therefore, there would be 25-30 days window between the introduction of the virus and the suspicion of disease. In Bulgaria, the prolonged HRP was seen, which was similar to the findings of the current investigation. While, in the domestic pig outbreaks in Estonia, the median HRP was 11 days.[26,27] As the length of HRP increases, there is an increase in the potential for virus propagation and spread. The possible reasons for this prolonged HRP are poor reporting, vague clinical signs, and poor awareness among the farmers.[27] The first fattening pig died of ASF on the farm, the other pigs died after 10 - 15 days and then, few pigs died daily or alternate days. This means that when the virus was introduced to the farm, only one fattening pig got infected. Most likely, the other pigs died later did not initially contact the disease, but rather did so through direct or indirect contact with infected fattening pigs. The majority of ASF outbreaks in endemic areas occur in backyard pig rearing due to the low bio-security measures at the farm.[27,28] Additionally, the majority of the farmers do not receive compensation when the animals die, causing them to suffer significant economic losses. To reduce the losses, farmers try to sell or slaughter the animals, when the disease is suspected. This further leads to the spread of the disease.[29] The swill feeding is also a very common practice in the pig farming in the area. Also, in the present outbreak, the hostel waste was being fed to the animals. Since the farm is in a close setting, the animals were not exposed to the stray and other animals. Similar to the previous report, the possible source of the present outbreak might be the anthropogenic factors, direct or indirect contact to contaminated fomites or food, entered the farm through human activities.[30] Through phylo-genetic analysis, the study was also aimed to gain insights into the genetic relationships and potential evolutionary

dynamics of Meerut virus in the Southeast Asian region. The genetic analysis revealed a distinct clustering of the Uttar Pradesh virus isolates from Vietnam with other Ib group isolates, indicating a close genetic relationship between these samples. Additionally, the mutant Chinese virus isolate showed clear genetic differences with the Vietnamese Ib group, confirming its suitability as an out-group for comparison. The *B646L* (p72) gene's sequencing and phylogenetic analysis have shown that ASFV genotype II is the cause of outbreaks in the northeast area, India.[31] The ASFVs circulating in Kerala, South India, and Mizoram, Northeast India, formed a distinct clade, according to the sequence analysis of the central variable region of the *B602L* gene, whereas ASFVs circulating in the Indian states of Arunachal Pradesh and Assam, India, belonged to a different clade.[32]

The findings from the present study provided valuable insights into the genetic diversity and relatedness of Uttar Pradesh virus isolates in the Southeast Asian region. This work further established the genetic relatedness between the ASFV circulating in this region and that in other regions of the country, marking the first research of the ASF outbreak in North India. Understanding the genetic makeup of these isolates is crucial for monitoring the potential spread and evolution of the virus, which can aid in the development of effective control and prevention strategies. Further studies with a larger dataset and additional geographical representation may enhance our understanding of the evolutionary dynamics of the virus.

This is the first report on the molecular characterization of the ASFV strains which may be circulating in North India. The phylogenetic studies revealed that the ASFV circulating in Uttar Pradesh, India, had a high genetic similarity with the Vietnam strains. Simultaneously, it showed genetic relationships and potential evolutionary dynamics in the Southeast Asian region. The slow spread of the disease among the pigs supports the moderate contagiosity of ASFV. It is important to be aware of all participants in the pig value chain since the anthropogenic component may be the likely source of disease at the farm.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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