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Molecular detection and characterisation of classical swine fever virus in Andhra Pradesh, India

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Abstract
An outbreak of Classical Swine Fever (CSF) was recorded in a back yard piggery farm in Bhimavaram mandal, West Godavari district of Andhra Pradesh, where mortality was observed in more than 20 pigs. The animals initially exhibited symptoms such as pyrexia, lethargy, and conjunctivitis. As the disease progressed, erythematic patches appeared on the abdomen, ears, and inside thigh regions, accompanied by tremors and circling movements just before death. Post-mortem examination revealed congestion and haemorrhages in vital organs and pneumonia in lungs. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) targeting 5' UTR, specific to *Pestivirus* was performed on three post-mortem samples, all of which tested positive. The PCR product was sequenced and compared using NCBI Nucleotide BLAST, which indicated 96% identity with Classical Swine Fever isolates. Phylogenetic analysis placed the PCR product close to CSF isolates under Subgroup 2.2 from Kerala, Uttar Pradesh and Mizoram states of India.

Keywords: Classical swine fever, polymerase chain reaction, phylogenetic tree, sequencing

Introduction
Classical Swine Fever (CSF), also known as Hog Cholera, is an economically significant and highly contagious disease recognized by the World Organisation for Animal Health (OIE) as notifiable. It is caused by an RNA virus classified under the *Pestivirus* genus of the *Flaviviridae* family, along with Bovine viral diarrhoea virus and Border disease virus [1].
Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is considered as the method of choice for detection of CSF due to its high sensitivity for detecting the virus even in the early stages of infection [2]. RT-PCR coupled with sequencing and phylogenetic studies based on the highly conserved 5' UTR gene enables differentiation from other *Pestiviruses* and classification of CSF genotypes or Sub groups [1, 3, 4, 5].

Materials and Methods
Case history: In January 2023, a backyard piggery farm in Bhimavaram mandal, West Godavari district of Andhra Pradesh, experienced pig mortality, with over twenty pigs dying within ten days. The animals displayed initial signs of lethargy and loss of appetite, along with pyrexia and conjunctivitis. Erythematic patches appeared on the abdomen, ears, and inner thigh regions. Death occurred two to three days after symptom onset, often accompanied by tremors and circling movements. Post-mortem examinations revealed congestion and haemorrhages in various organs. Pneumonia was observed in lungs. Based on symptoms and lesions, Classical Swine Fever was suspected and post-mortem samples like Lung, Spleen and lymph node were sent to the Veterinary Biological and Research Institute (VBRI) for laboratory confirmation.

Detection of CSFV by RT-PCR: Total RNA was isolated from tissues using TRIzol (Invitrogen, Thermo Fisher) and one-step RT-PCR was performed using the Prime Script™ One Step RT-PCR Kit Ver.2 (Dye plus) by Takara Bio. The RT-PCR targeted the 5'UTR end of *Pestivirus* [6] using the following primers:

Pestivirus 324 Forward: 5'-TGCCCTTAGTAGGACTAGCA-3'
Pestivirus 326 Reverse: 5'-CAACTCCATGTGCCATGTAC-3'
RT-PCR reaction volumes included 25 µl of 2X Master mix, 1 µl each of Primer pair (20 pm/µl), 2 µl of Superscript Taq enzyme, 1 µl of the template, and 20 µl of Nuclease-free water, making a total volume of 50 µl. The thermal profile involved steps such as CDNA preparation at 50 °C for 30 min, initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 45 sec, annealing at 57 °C for 30 sec, extension at 72 °C for 30 sec, for 39 cycles with a final extension at 72 °C for 7 min. The PCR products were electrophoresed on a 1.5% agarose gel in 1X TBE buffer with 0.5 µg/ml of ethidium bromide and visualized under UV illumination.

Sequencing and Phylogenetic analysis: For virus characterization, one RT-PCR product encoding the 5' UTR gene was sequenced using Sanger sequencing. Chromatograms were analyzed with Chromas software version 2.6.6, and sequence errors were corrected. The contig sequence was aligned using Codon code aligner and subjected to NCBI nucleotide blast for virus confirmation. phylogenetic analysis was performed by aligning reference sequences of various CSFV subgroups and other pestivirusesusing Clustal W program in MEGA 11 software. The phylogenetic tree was constructed using Neighbour-joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the CSF isolate (VBRI, AP).

Results and Discussion

Classical Swine Fever is one among the five major livestock diseases affecting swine population in India [7]. Due to its devastating effect on pig industry, timely detection of the disease is of paramount importance. Early detection helps in controlling the disease thereby reducing huge economic loss. In the current outbreak, Primary investigation based on clinical signs and post-mortem findings as shown in Image1, suggested CSF as the cause of the outbreak, aligning with the OIE manual [8] and previous reports by Malik *et al*. 2020 [2]; Ravishankar *et al*. 2007 [9].

Image 1: Erythematic patches on skin and Pneumonia in lungs

According to Malik *et al*. 2020 [2], CSF was initially reported in Andhra Pradesh and has not been recorded since. This is the first CSF epidemic in Andhra Pradesh to be confirmed by RT-PCR. As Reverse transcriptase PCR is considered an appropriate emerging strategy for confirmation of clinical cases due to its high sensitivity and rapidity [7], Presently RT-PCR was conducted targeting 5' UTR region of *Pestivirus group* yielding a 288 bp amplicon which was specific to the designed primers (Image 2) as reported by Vilcek *et al*. 1994 [6] confirming the presence of a pestivirus. As pigs are commonly infected by all members of Pestiviruses like CSF, Bovine Viral Diarrhea (BVD) and Border Disease (BD), specific and accurate detection of CSF and its differentiation from BVD and BD is important to take necessary control measures like vaccinations [10]. As per Mosena *et al*., 2020 [3], Anita *et al*., 2020 [10] and Kamnboj *et al*., 2022 [5] "Conserved genomic regions especially 5' UTR can be used for genotyping *Pestivirus* species and variants through phylogenetic analysis". Hence the PCR product was sequenced to distinguish the type of *Pestivirus*.

When the sequence (VBRI AP) was analyzed by NCBI Blast, it showed 96% identity with Indian and other south East Asian CSF isolates confirming the presence of CSF virus in the sample tested.

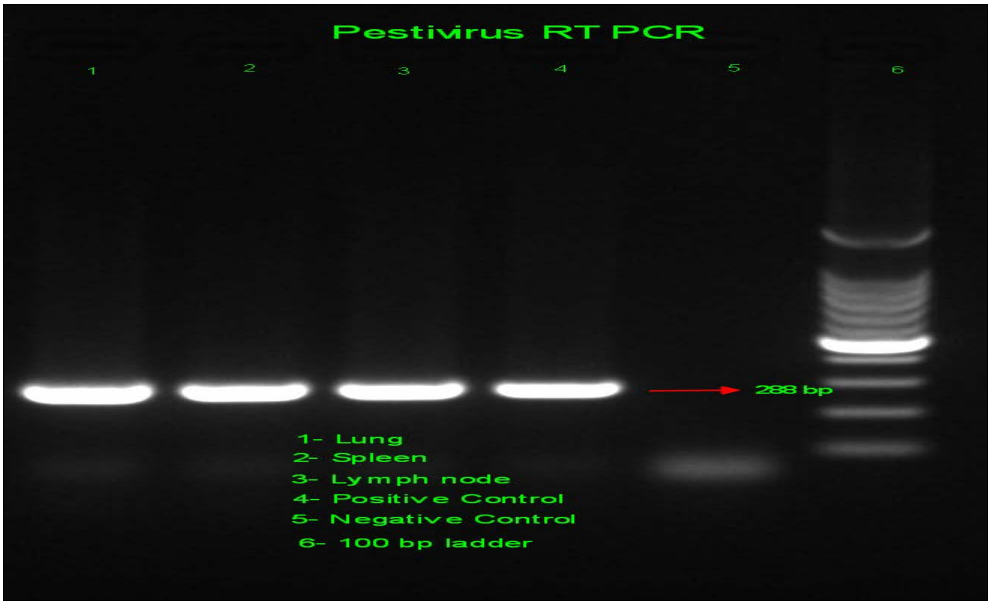

Image 2: RT PCR for Pestivirus targeting 5'-UTR with product size of 288 bp

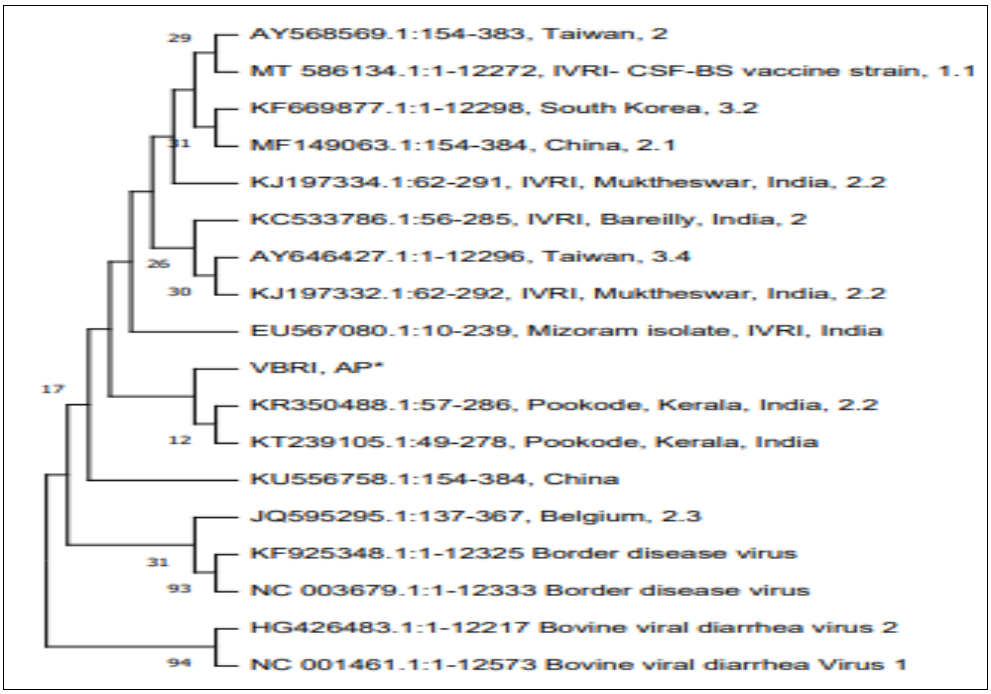

Fig 1: Phylogenetic tree is based on the 5'-UTR nucleotide sequence. Evolutionary analysis was conducted in MEGA 11 Neighbour-joining method with bootstrap consensus tree inferred from 1000 repetitions. The sample VBRI AP in our study is marked with a black asterisk.

Phylogenetic analysis was done using the 5' UTR region that was sequenced as shown in figure 1. 5' UTR region is one of the three genomic locations acknowledged for its ability to categorize CSFV isolates, determine genetic connections, and position them on phylogenetic trees [2]. CSF virus is sub divided in to three phylogenetic groups (Group 1, 2 and 3) which is further divided into sub groups. In India previous published genotyping reports revealed genotype 1.1 prevalence. Currently there is increasing evidence and co circulation of genotype 2.2 followed by 2.1 [2]. The phylogenetic analysis of current outbreak sample VBRI AP showed grouping with Indian isolates in sub group 2.2, from Kerala, Uttar Pradesh and Mizoram states of India which is in accordance with r Bhaskar *et al*. 2015 [7], who reported that sub group 2.2 is the most predominant CSF serotype circulating in South Indian states. The CSF vaccine currently in use in the country (CSF BS) belongs to subgroup 1.1 and grouped separately from the isolate. In the existing scenario of increasing number of isolates from subgroup 2.2 in the country, the efficacy of the vaccine from subgroup 1.1 needs to be studied.

When compared with CSF isolates from other countries isolates from China and Taiwan grouped closely than with isolates from South Korea and Belgium. Other *Pestiviruses* like BVD 1, BVD 2 and Border Disease virus branched separately confirming that the isolate is CSF and not any other pestivirus.

Conclusion

The cause for current outbreak is confirmed as Classical swine Fever. Sequencing and phylogenetic analysis showed that the virus is similar to Indian CSF isolates in the subgroup 2.2. As part of necessary control measures, field veterinarians were advised to conduct CSF vaccination and provide symptomatic treatment for affected pigs in the farm.

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