RESEARCH ARTICLE

Molecular Epidemiology, Seroprevalance, Serum Biochemical Alterations and *In Silico* Analysis of African Swine Fever Virus in Karnataka

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Abstract

African swine fever (ASF) is a highly contagious and haemorrhagic transboundary animal disease affecting swine, characterized by a significant morbidity and mortality rate. Comprehensive understanding of ASF necessitates studying its pathology, pathogenesis, and molecular characterization of individual ASF virus isolates. Current study aimed to investigate the seroprevalence, molecular detection, biochemical alterations in the affected pigs, nucleotide sequencing and *In silico* analysis of the ASF isolates from southern regions of Karnataka. ASF outbreaks (n=5) occurred in Karnataka between 2023 and 2024, resulting in substantial losses for farmers. Indirect ELISA testing revealed 15.22% (28/184) of serum samples tested positive for ASFV antibodies. ASFV confirmation was achieved through PCR targeting the p72 gene, identifying 11 positive cases out of 15 tested. Two strains of ASF were isolated from Karnataka. Nucleotide sequencing and *In silico* analysis revealed two isolates from Karnataka phylogenetically similar to the West Bengal isolate. In addition, predicted protein belongs to the Template 6l2t.1.A B646L, Major capsid protein of ASF virus major capsid protein p72 gene with complete sequence identity.

Key words: African swine fever, Biochemical parameters, *In-silico* analysis, Molecular Epidemiology, Seroprevalence.

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Introduction

India›s pig population, totaling 9.06 million, representing 1.7% of the country›s total livestock (DADF, 2019 https://dahd.nic.in/), has significantly contributed to the meat industry, producing 8.11 million tonnes of meat and marking a 6% increase from the previous year. Pigs are exposed to several bacterial and viral infectious illnesses that afflict serious risk to pork production. African swine fever virus (ASFV) is one of those diseases that have been a major source of concern for the pig industry (VanderWaala and Deena, 2018). Spread of diseases are associated with lack of well-defined production practices, structured breeding programs, pricey feed, insufficient marketing systems, financial constraints, inability to obtain high-quality piglets, a lack of knowledge about scientific pig farming methods, weak biosecurity controls, and unofficial trade activities and a lack of timely access to healthcare and vaccination facilities especially among those from socioeconomically disadvantaged backgrounds (Jothika *et al.,* 2022).

ASFV, a large, complex, cytoplasmic, icosahedral double-stranded DNA virus, is the causative agent of ASF (Dixon *et al*., 2005). It affects animals that belong to the Suidae family, and *Ornithodoros* ticks (Patil *et al.,* 2020). ASF is a viral haemorrhagic disease, which kills domestic pigs and Eurasian wild boar with remarkably high fatality rates. Although its host range is small and it does not present a health danger to humans (zoonotic potential), it has a significant socioeconomic influence on a wide variety of stakeholders of pig industry. Consequently, it is regarded as a notifiable disease by the World Organization for

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Animal Health (WOAH) (Blome *et al.,* 2020). Regarding the ASF outbreak, In India, the first known outbreak of ASF was studied and reported in 2021 (Rajukumar *et al.,* 2021). Nevertheless, in Karnataka no outbreak of ASF was recorded until 2023. In southern India, first ASF outbreak in Kerala was reported by Hiremath *et al.* (2023). It is for the first time, present research work was carried out in the southern districts of Karnataka focused on the molecular confirmation, serum biochemical alterations, epidemiology and seroprevalence of ASFV in the affected pigs.

Materials and Methods

The present work was carried out in Veterinary College, Hassan in cooperation with IAH and VB Hebbal, Bengaluru (India) with the prior CCSEA approval (No. HVC/IAEC/11/2023). The five outbreaks of the ASF that occurred between August 2023 and February 2024 in the southern Karnataka, *viz.*, Chikkaballapur (13.43°N 77.72°E), Hoskote (13.07°N 77.8°E) in Bangalore Rural District, Mangalore (12.91°N 74.85°E) in Dakshina Kannada district, Hassan (12.86°N 75.98°E) and Saalu hunase in Bengaluru Urban District (12.81°N 77.51°E) were investigated.

Sampling and Blood Collection

The basic random sampling approach, as detailed in Thrusfield (2008), was used to calculate the sample size for seroprevalence. Numbers of piggery farms in the region›s southern districts were visited, with thorough questionnaire to collect detailed data from every pig farm, including the age and gender of each animal, breed, size of the herd, number of infected animals, and animal history etc. Blood samples were collected from ear veins into vacutainers equipped with a clot activator across various districts from multiple sources: pig farms (167 samples) and slaughterhouses (17 samples), and serum was stored at -20°C for further analysis. Out of 184 samples, 76 were collected from disease outbreak area.

Seroprevalance of ASFV using Indirect ELISA and Serum Biochemical Parameters

The ID Screen® African Swine Fever Indirect ELISA kit from France was utilized; which contains 96-well microplate with precoated with p32, p62 and p72 ASFV recombinant proteins reference positive and negative blood samples along with an anti-ASFV HRPO conjugate. Serum samples were screened using kit as per the manufacturer's instructions. Serum biochemical parameters, like, SGPT, SGOT, creatinine, and urea, were analyzed using an automated serum analyzer with the available commercial kits.

Molecular Confirmation of ASFV

DNA extraction from pooled tissue samples was carried out using the HiMedia kit as per the manufacturer's instructions followed by PCR. Amplification of the p72 gene of ASFV was carried out by using Forward primer ‹AGTTATGGGAAACCCGACCC› 20 bp, Reverse primer 'CCCTG AATCGGAGCATCCT' 19 bp (Agüero *et al.,* 2003). The amplicon size of 257 bp was visualized under 2% agarose gel electrophoresis. The PCR cyclic conditions used were initial denaturation at 95°C for 10 min, followed by denaturation at 95°C for 15 sec, annealing at 62°C for 30 sec, extension at 72°C for 30 sec, which was repeated for 45 cycles, and final extension at 72°C for 7 min, lastly it was held at 4°C. The gel extraction of the PCR product was completed consequently; sequencing of the PCR product was carried out commercially at Eurofins, Bengaluru along with the respective primers (10 mM), for sequencing with detailed instructions provided.

In Silico Analysis of Sequence Data

In silico Analysis of Sequence Data of p72 gene was carried out using various bioinformatic tools and several online available algorithms. GenBank database were used in order to conduct a comparative sequence analysis. Sequences from other origin nations as well as India were included.

Statistical Analysis

Statistical analysis was conducted using GraphPad Prism statistical software. Mean values and standard errors were calculated for various parameters. The data on biochemical parameters were analyzed using a t-test.

Results and Discussion

Seroprevalence of ASFV using Indirect ELISA

Among the 184 serum samples subjected for the analysis, 28 tested positive, indicating 15.22% of the samples contained ASFV antibodies. The kit has a sensitivity of 93% and a specificity of 100% (Carlson *et al.,* 2018). These parameters were applied to estimate the true prevalence of ASF in the southern regions of Karnataka by using formula TP= (AP+Sp-1) / (Se+Sp-1), where TP is true prevalence, AP is apparent prevalence, Sp is test specificity, and Se is test sensitivity. With an apparent prevalence of 15.22% (0.1522), a sensitivity of 93% (0.93), and a specificity of 100% (1.0), the true prevalence approximates 16.37 %. Therefore, ASF›s true prevalence in southern Karnataka was about 16.37%. This finding concurred with the earlier report on ASF prevalence of about 16.9% in Senegal using ELISA testing (Etter *et al*., 2011). Remarkably, ASF prevalence tends to be higher during the dry season due to the increased presence of ASFV-positive ticks (Awosanya *et al*., 2015). Furthermore, out of 28 positive animals for ASF antibodies, 16 were male pigs (57.14%) and 12 female pigs (43.86%).

Figure 1 illustrates the district-wise occurrence of ASF in southern Karnataka, which varied from 0.0% to 66.67%. No seropositivity was found in the samples collected from disease free districts. Out of 76 sera samples collected from outbreak area, 28 samples were found positive for ASF antibodies, resulting in a positivity rate of 36.84% in the affected area. This could be attributed to the possibility

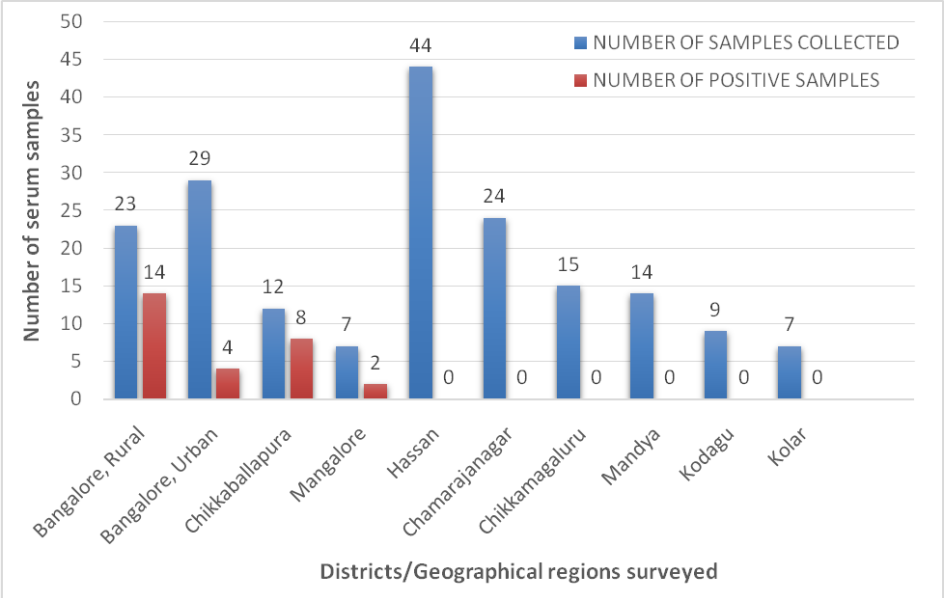

Fig. 1: District-wise geographical distribution of ASF in southern Karnataka

that the disease may have caused mortality in infected animals before they could develop antibodies against ASF. Furthermore, seroconversion may have occurred in the last stages of illness in the animals that survived.

Regarding age, there were 18 positive cases detected by ELISA in pigs below 6 months of age (9.7%, 18/184), 3 in the 6 months to 1-year age group (1.6%, 3/184), and 7 in pigs more than 1 year (3.80%, 7/184). After natural infection, pigs typically develop antibodies against ASFV within 7-10 days. Pregnant and nursing sows also experience immunological suppression, which increases their vulnerability to ASF, with lower virus doses being enough to cause infection, as reported by Nurmoja *et al.* (2020). Oh *et al.* (2021) found that weaned pigs exhibited significantly higher ASF viral loads in their lungs, liver, lymph nodes, and blood compared to fattening pigs and sows. This is also in accordance with the current findings of 9.7% positive cases in piglets below 6 month which could be attributed to the weak immune status in the younger piglets than the adults.

Serum Biochemical Parameters

Serum biochemical parameters like BUN and serum creatinine levels showed significant ($p<0.05$) differences between sick and control pigs. On the other hand there were highly significant ($p<0.01$) variations in serum enzymes like AST and ALT levels, the levels being higher in diseased than in healthy control pigs (Table 1). Significant variation in the AST and ALT is mainly attributed to the liver affections or hepatitis in the infected pigs. The elevated serum creatinine and BUN levels reflected renal damage in the ASF affected animals. Walczak *et al.* (2021) corroborated the observation that diseased mice had noticeably higher levels of AST and ALT enzymes, a sign of liver damage.

Table. 1: Mean (±SE) values of serum biochemical parameters in control and ASF-positive pigs

Biochemical parameters	Control (n=6)	Diseased (n=10)
Creatinine (mg/dL)	0.93± 0.07	1.21± 0.15*
AST (U/L)	46.36± 3.96	81.47± 5.24**
ALT(U/L)	35.92±3.41	54.76± 3.76**
BUN (mg/dL)	23.76±1.78	27.50± 3.07*

*p<0.05, **p<0.01

Molecular Detection of ASF by Polymerase Chain Reaction

PCR of tissue samples (15), including palatine tonsil, lung, heart, liver, kidney, and lymph node, collected from pigs in regions with suspected disease outbreaks and increased mortality, targeting p72 gene revealed 11 samples positive with an observed amplicon size of 257 base pairs (Fig. 2) resulting in a positive rate of 73.33% in the current study. Aguero *et al.* (2003) utilized conventional PCR of p72 gene to detect ASFV in early clinical samples.

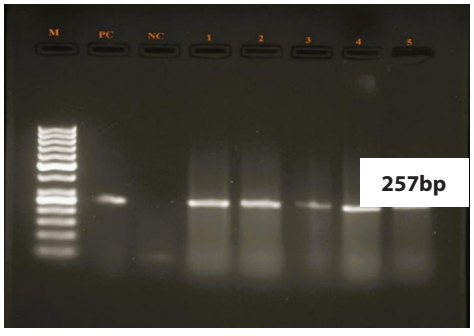

Fig. 2: Tissue samples showing positive for p72 gene of ASF in PCR. Lane M: 50bp DNA ladder, Lane PC: Positive control, Lane NC: Negative control, Lane 1-5: Tissue samples

Table 2: Bioinformatics analysis of nucleotide of 257 bp of p72 gene of ASFV and predicted protein sequence of about 84 amino acids

ASF Isolate from Hoskote–Nucleotide Sequence Karnataka-India (Lat 12.9556°N, Long 77.8155°E)
TATGGGAAACCCGATCCCGAACCCACTTTGAGTCAAATCGAAGAAACACATTTGGTGCATTTTAATGCGCATTTTAAGCCTTATGTTCCAGTAGGGTTT GAATACAATAAAGTACGCCCGCATACGGGTACCCCCACCTTGGGAAACAAGCTTACCTTTGGTATTCCCCAGTACGGAGACTTTTTCCATGATATG GTGGGCCATCATATATTGGGTGCATGTCATTCATCCTGGCAGGATGCTCCGATTCAGGG
ASF Isolate from Hoskote Predicted Protein Sequence Karnataka-India 84 Amino acids
YGKPDPEPTLSQIEETHLVHFNAHFKPYVPVGFEYNKVRPHTGTPTLGNKLTFGIPQYGDFFHDMVGHHILGACHSSWQDAPIQ
ASF Isolate from Chikkaballapura – Nucleotide Sequence Karnataka-India (Lat 13.389385°N, Long 77.715698°E)
TATGGGAAACCCGATCCCGAACCCACTTTGAGTCAAATCGAAGAAACACATTTGGTGCATTTTAATGCGCATTTTAAGCCTTATGTTCCAGTAGGGTTT GAATACAATAAAGTACGCCCGCATACGGGTACCCCCACCTTGGGAAACAAGCTTACCTTTGGTATTCCCCAGTACGGAGACTTTTTCCATGATATG GTGGGCCATCATATATTGGGTGCATGTCATTCATCCTGGCAGGATGCTCCGATTCAGGG
ASF Isolate from Chikkaballapura Predicted Protein Sequence-Karnataka-India-84 Amino acids
YGKPDPEPTLSQIEETHLVHFNAHFKPYVPVGFEYNKVRPHTGTPTLGNKLTFGIPQYGDFFHDMVGHHILGACHSSWQDAPIQ

In silico Analysis of p72 Gene

The sequenced nucleotide of 257 bp of p72 gene of ASFV and predicted protein sequence of about 84 amino acids was subjected for various bioinformatics analysis using several methods, which gave valuable information about the capsid protein of p72 region of the ASFV as shown in Table 2.

QMEAN is a composite estimator based on different geometrical properties and provides both global (*i.e.* for the entire structure) and local (*i.e.* per residue) absolute quality estimates on the basis of one single model (Benkert *et al*., 2011). For Swiss-Models' Homology, QMEAN have been calculated during the normal modelling pipeline process. Predicted ASFV protein belongs to the Template 6l2t.1.A B646L, Major capsid protein, African swine fever virus major capsid protein p72 gene with 100% sequence identity (Fig. 3, 4) as depicted in the graph with QMEANDisCo global score of 0.40 ± 0.05 upon analysis (Studer *et al*., 2020). The global and per-residue model quality has been assessed using the QMEAN scoring function.

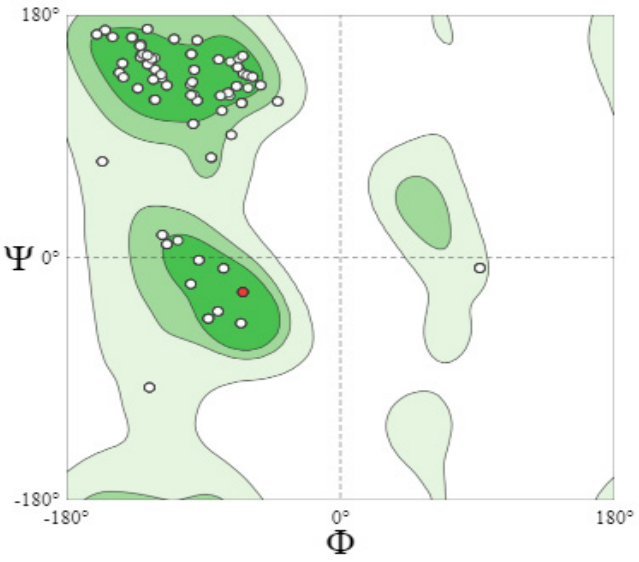

Fig. 3: Ramachandran plot analysis of predicted protein sequence

Fig. 4: Alignment of predicted protein sequence suggesting its 100% identity with 6l2t.1.A B646L Major capsid protein African swine fever virus major capsid protein p72 gene

Model-Template Alignment of predicted protein sequence suggests that there is highly Low SOA (Solvent Accessibility) and Lower confidence gradient in the predicted protein sequence (Studer *et al*., 2020). Predicted 3D protein model generated using https://swissmodel. expasy.org/ interactive/ e2rcwY/models/ in the present study is shown in Fig. 5. Models were built based on the target-template alignment using ProMod3 (Studer *et al*., 2020). Coordinates which were conserved between the target and the template were copied from the template to the model. Insertions and deletions were remodeled using a fragment library. Side chains were then rebuilt. Finally, the geometry of the resulting model was regularized by using a force field.

Phylogenetic Analysis

Phylogenetic analysis of ASFV isolates focused on a 257-nucleotide segment of the p72 gene, using sequences from global strains, aligned against 21 other strains retrieved from GenBank to construct Figure 6 & 7.

The p72 gene sequences from ASFV isolates in Chikkaballapura and Hoskote, Karnataka, showed divergence from West Bengal (WB) isolates but shared 100% sequence similarity with WB and BALI isolates. The initial ASF outbreak in India began in Northeast India, a likely source of the Karnataka pandemic (Rajukumar *et al.,* 2021). Movement of pigs from northeastern to southern regions for sale is a significant risk

Fig. 5: 3D modeling of the predicted protein sequence of the ASFV p72 gene

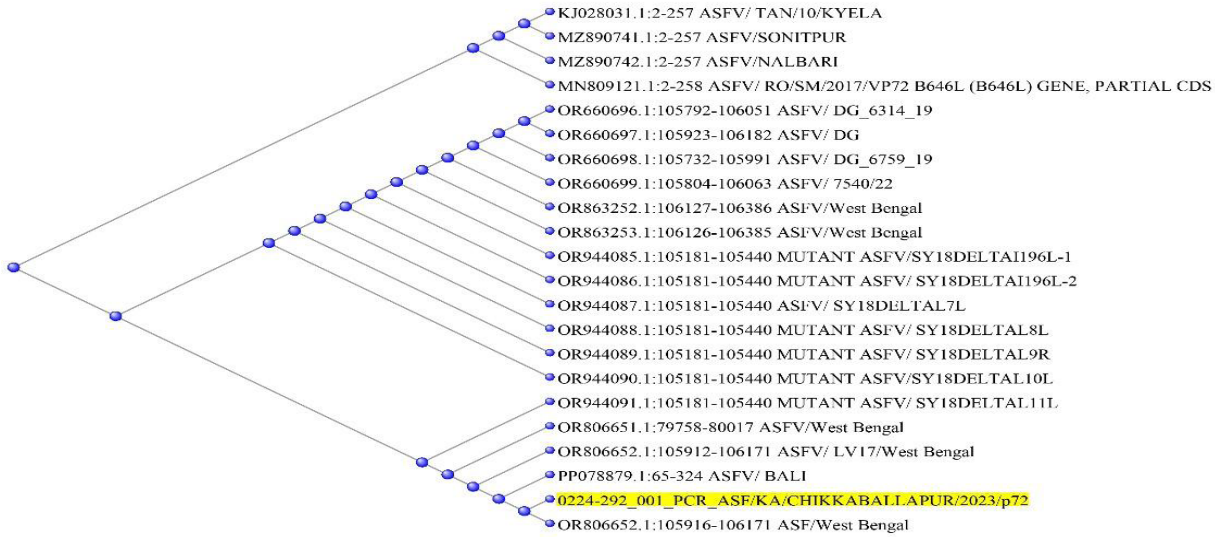

Fig. 6: Phylogenetic tree/cladogram based on 257 nt sequence data-set from p72 genomic regions of various Indian isolates of ASFV compared with Chikkaballapura isolate

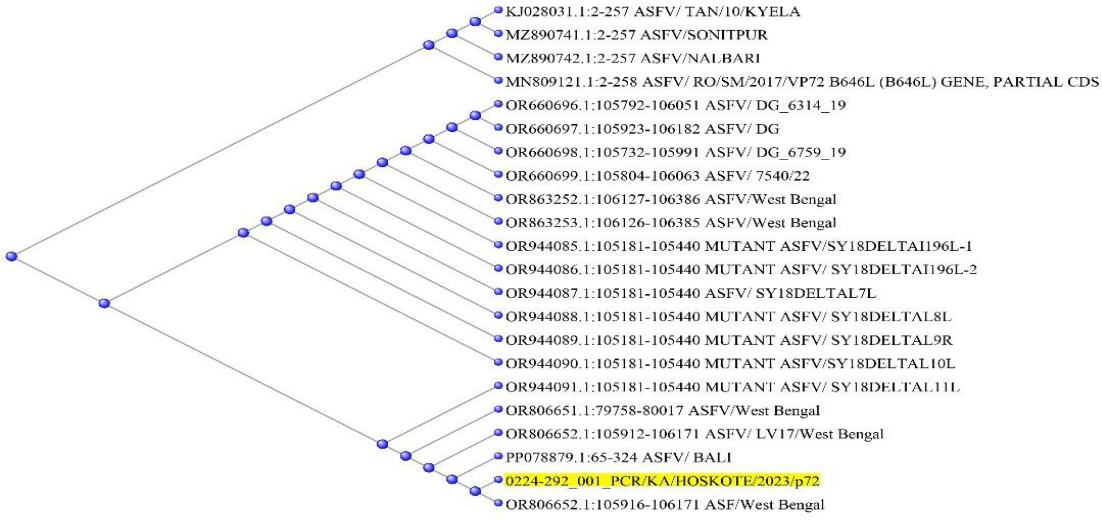

Fig. 7: Phylogenetic tree/cladogram based on 257 nt sequence data-set from p72 genomic regions of various Indian isolates of ASFV compared with Hoskote isolate

factor for ASF spread in Karnataka, exacerbated by practices like swill feeding, non-scientific farming methods, and limited farmer awareness. This study highlights phylogenetic analysis as a valuable epidemiological tool for investigating ASF outbreaks in Karnataka.

Conclusions

This study represents the first comprehensive investigation of ASF outbreaks including its seroprevalence in the southern regions of Karnataka. Serum biochemical analysis revealed significant differences in AST and ALT values. The PCR using p72 gene was found to be highly sensitive and reliable for the early diagnosis of ASF infections. Furthermore, the phylogenetic analysis indicated that the ASF isolate from Karnataka shares a similar sequence with the strain from the West Bengal outbreak. Predicted protein of African swine fever virus major capsid protein p72 gene belongs to the Template 6l2t.1.A B646L, Major capsid protein with complete identity. Future research could expand genomic investigations to deepen our understanding of disease dynamics, group interactions, and transmission patterns nationwide.

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Conflict of Interest: Authors express no conflict of interest in this research.

References

Aguero, M., Fernández, J., Romero, L., Sánchez Mascaraque, C., Arias, M., & Sánchez-Vizcaíno, J.M (2003). Highly sensitive PCR assay for routine diagnosis of African swine fever virus in clinical samples. *Journal of Clinical Microbiology, 41*(9), 4431-4434.

Awosanya, E. J., Olugasa, B., Ogundipe, G., & Grohn, Y. T. (2015). Sero-prevalence and risk factors associated with African swine fever on pig farms in southwest Nigeria. *BMC Veterinary Research*, *11*, 1-11.

Benkert, P., Biasini, M., & Schwede, T. (2011). Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics*, *27*(3), 343-350.

Blome, S., Franzke, K., & Beer, M. (2020). African swine fever - A review of current knowledge. *Virus Research*, *287,* 198099.

Carlson, J., Zani, L., Schwaiger, T., Nurmoja, I., Viltrop, A., Vilem, A., ... & Blome, S. (2018). Simplifying sampling for African swine fever surveillance: Assessment of antibody and pathogen detection from blood swabs. *Transboundary and emerging diseases*, *65*(1), e165-e172.

Chen, V.B., Arendall, W.B., Headd, J.J., Keedy, D.A., Immormino, R.M., Kapral, G.J., ... & Richardson, D.C. (2010). MolProbity: All-atom structure validation for macromolecular crystallography. *Acta Crystallographica Section D: Biological Crystallography*, *66*(1), 12-21.

Dixon, L.K., Escribano, J.M., Martins, C., Rock, D.L., Salas, M.L., & Wilkinson, P.J (2005). Asfarviridae. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A., editors. *Virus Taxonomy. 8th Report of the International Committee on Taxonomy of Viruses*. Elsevier Academic Press, London, p. 135-143.

Etter, E.M., Seck, I., Grosbois, V., Jori, F., Blanco, E., Vial, L., ... & Roger, F.L. (2011). Seroprevalence of African swine fever in Senegal-2006. *Emerging Infectious Diseases*, *17*(1), 49.

Hiremath, J., Hemadri, D., Nayakvadi, S., Kumar, C., Gowda, C. S. S., Sharma, D., ... & Gulati, B.R. (2024). Epidemiological investigation of ASF outbreaks in Kerala (India): Detection, source tracing and economic implications. *Veterinary Research Communications*, *48*(2), 827-837.

Jothika, C., Mohanapriya, M., Balasubramanyam, D., Senthilkumar, K., Devaki, K., & Kathiravan, R.S. (2022). Feeding practices adopted by small holding pig farmers in Tamil Nadu. *Journal of Krishi Vigyan*, *11*(si), 43-46.

Nurmoja, I., Mõtus, K., Kristian, M., Niine, T., Schulz, K., Depner, K., & Viltrop, A. (2020). Epidemiological analysis of the 2015-2017 African swine fever outbreaks in Estonia. *Preventive Veterinary Medicine*, *181*, 104556.

Oh, T., Do, D. T., Lai, D. C., Nguyen, T. C., Van Vo, H., & Chae, C. (2021). Age-related viral load and severity of systemic pathological lesions in acute naturally occurring African swine fever virus genotype II infections. *Comparative Immunology, Microbiology and Infectious Diseases*, *79*, 101709.

Patil, S.S., Suresh, K.P., Vashist, V., Prajapati, A., Pattnaik, B., & Roy, P. (2020). African swine fever: a permanent threat to Indian pigs. *Veterinary World*, *13*(10), 2275.

Rajukumar, K., Senthilkumar, D., Venkatesh, G., Singh, F., Patil, V.P., Kombiah, S., ... & Singh, V.P. (2021). Genetic characterization of African swine fever virus from domestic pigs in India. *Transboundary and Emerging Diseases*, *68*(5), 2687-2692.

Studer, G., Rempfer, C., Waterhouse, A. M., Gumienny, R., Haas, J., & Schwede, T. (2020). QMEAND is Co-distance constraints applied on model quality estimation. *Bioinformatics*, *36*(6), 1765-1771.

Thrusfield, M. (2018). *Veterinary Epidemiology*. John Wiley & Sons.

Vander Waal, K., & Deen, J. (2018). Global trends in infectious diseases of swine. *Proceedings of the National Academy of Sciences*, *115*(45), 11495-11500.

Walczak, M., Wasiak, M., Dudek, K., Kycko, A., Szacawa, E., Olech, M., ... & Szczotka-Bochniarz, A. (2021). Blood counts, biochemical parameters, inflammatory, and immune responses in pigs infected experimentally with the African swine fever virus isolate Pol18_28298_O111. *Viruses*, *13*(3), 521.