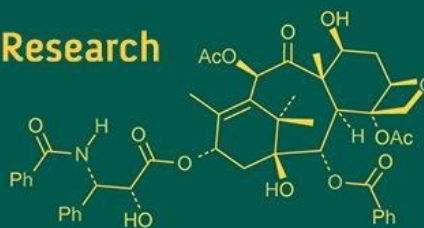


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Assessment of antibody prevalence against African swine fever virus in an Indian pig population

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Abstract

African swine fever (ASF) is an economically devastating viral disease of pigs caused by the sole member of *Asfarviridae* family i.e. African swine fever virus (ASFV). The rapid global spread of ASF has put the pig industry in grave danger due to lethal nature of the disease. The current study reports presence of ASFV antibodies in the state of Uttar Pradesh based on the data generated through screening of serum samples in ID Screen® African swine fever Competition ELISA kit and E-lab Science ASF Competition ELISA kit. Repeated ASF outbreaks in certain Indian states need to be tackled by using available diagnostics. Thereby, such a screening test for ASF could be quite helpful in the absence of any such indigenous assay or kit in Indian scenario.

Keywords: African swine fever, ASFV antibodies, commercial kit

1. Introduction

African swine fever (ASF) is a lethal, highly contagious and often fatal hemorrhagic disease affecting domestic and wild pigs. The causative agent, African swine fever virus (ASFV), is a large and complex double-stranded DNA (dsDNA) virus with a genome size ranging from approximately 170 to 194 kbp, encoding nearly 60 structural and 100 non-structural proteins (Alonso *et al.*, 2018) ^[1]. ASFV is the sole member of family *Asfarviridae* and shares several biological characteristics, including genome organization and cytoplasmic replication, with other nucleo-cytoplasmic large DNA viruses (NCLDV) such as poxviruses and iridoviruses (Tu *et al.*, 2018) ^[22]. The clinical outcome of ASF varies considerably depending on the virulence of the infecting ASFV strain. Disease manifestations may range from mild, nonspecific signs such as transient fever and anorexia to severe acute form of disease associated with high fever and almost 100% mortality with characteristics such as fever, cyanosis, lethargy, ecchymotic haemorrhages, respiratory and gastro-intestinal disturbances, and dullness with death typically occurring within 3-8 days post-infection (Dixon *et al.*, 2019) ^[3]. Moderately virulent strains induce similar signs, but more severe haemorrhages as compared to acute form with mortality rates between 30% and 70%. In contrast, infection with low-virulent strains results in chronic form of ASF, marked by skin lesions, intermittent fever, multifocal cutaneous necrosis, secondary bacterial infections, and arthritis (Gallardo *et al.*, 2015) ^[7]. In the year 1909 (Kenya), a typical fever affecting only pigs with short course of disease and nearly 100% death rate was described for the first time. Eventually, similar outbreaks were reported from Tanzania (1910) and Zambia (1912). Earlier, the disease was confused with that of hog cholera/classical swine fever due to its prior existence. Thereby, the first report of African swine fever was published in the year (1921), Montgomery. The probable source of ASF outbreak was attributed to the cycle between warthogs, *Ornithodoros* soft ticks and pigs. Although, it remained confined within the African continent causing outbreaks in different regions periodically until 1957. Then, the disease entered Portugal via contaminated food waste and illegal transport (Gaudreault *et al.*, 2020) ^[11]. Within the next decade, ASF spread from Portugal to other parts of Europe, Caribbean nations and Brazil. Even though some nations tried to eradicate ASF with stringent policies, but they failed to do so. The disease was then re-introduced into Georgia (2007) followed by Russia, Ukraine, Baltic and Caucasus (Penrith and Kivaria, 2022) ^[19].

After 2010, ASF spread rapidly across Eurasia with first outbreak reported from China in 2018 and nearby 20 other nations. In India, ASF cases were seen initially during January, 2020 from Assam and Arunachal Pradesh but the first report was confirmed in May, 2020 (WOAH, 2023) [24]. Till date, ASF has been reported in nearly 22 out of 28 Indian states. In the past few years, cases have been rising at a faster pace which is a matter of concern for our country's pig population.

Control of ASF in affected regions primarily depends on early detection, strict quarantine, stamping-out policies, strict on-farm security, surveillance monitoring, controlled movements and efficient sterilization procedures (Liu *et al.*, 2023) [14]. Virological tests include detection or identification of ASFV antigens. Virus isolation and haemadsorption test (HAD) are the classical methods for virus identification. Additionally, these are reference tests preferred in case of primary outbreaks (Gallardo *et al.*, 2015) [7]. Although, it is time-taking, requires special lab set-up and availability of trained personnel. Since, last two decades non-HAD strains of virus are circulating rampantly making it difficult to perform these tests. Molecular assays for ASFV genome detection are routinely employed for early detection that result in high sensitivity and specificity (Sanchez-Vizcaino *et al.*, 2006) [6]. Various types of Polymerase chain reaction (PCR) viz, Real-time PCR, conventional PCR and multiplex PCR along with isothermal amplification tests have been used widely. Real time PCR assays developed by King *et al.* (2003) [13] and Fernandez-Pinero *et al.* (2013) [4] have been considered as 'gold standard' tests by WOAH for the detection of ASFV genome and is being used in all reference labs. Similarly, for ASF virus detection FAT is quite useful in samples such as tissue smears or leucocyte cultures by using fluorescent microscope. The additional benefit in FAT is the detection of non-HAD ASF virus strains that is indistinguishable in HAD test (Oura *et al.*, 2013) [17]. Direct immunoperoxidase, immunohistochemistry, and antigen ELISAs can also be used for ASF antigen detection.

However, serological surveillance plays a critical role in confirming disease pattern following outbreaks and in identifying animals infected with low-virulence ASFV strains. In recent years, numerous naturally attenuated and modified ASFV strains have been reported in ASF-endemic regions which produce mild, subclinical infections, allowing viral circulation to remain undetected thus facilitating disease spread (Onyilagha *et al.*, 2024) [16]. The absence of evident clinical signs, along with low and intermittent viremia, can compromise the sensitivity of molecular diagnostic assays, whereas serological methods are more effective in identifying such infections. ASFV-specific antibodies typically become detectable within 7-10 days post-infection (dpi) and may persist for prolonged periods, in some cases for several years (Gallardo *et al.*, 2015) [7]. Accordingly, the presence of anti-ASFV antibodies serves as a reliable marker of current or past infection. In this context, indirect/blocking ELISAs can be very helpful (Watanabe *et al.*, 2023) [23].

Infected animals mount strong antibody responses against several highly conserved ASFV structural proteins, including p30, p54, and p72, making these antigens particularly suitable for serological assay development

(Cubillos *et al.*, 2013; Gallardo *et al.*, 2009; Jia *et al.*, 2017) [2, 8, 12]. A number of ELISAs targeting ASFV-specific antibodies have been developed and commercialized worldwide. However, none of the currently available commercial ELISA kits are manufactured in India. Therefore, the present study describes the use of two commercial kits (ID Screen® African swine fever Competition ELISA kit, France and E-lab Science ASF Competition ELISA kit, China) for the detection of anti-ASFV antibodies in porcine serum samples in the state of Uttar Pradesh, India. India have around 9.06 million in the 2019 Livestock Census, with Uttar Pradesh (UP) holding a significant share, having approximately 0.41 to 0.49 million pigs, making it a key state for pig farming alongside Assam and Jharkhand. So that the generated data can play a major role to strengthen the preparedness efforts to tackle ASF in India.

2. Material and Methods

2.1 Serum samples collection

Porcine samples (n = 330) were collected from pigs present in college farm and districts of Uttar Pradesh in accordance with ethical guidelines. The samples were collected between April to December, 2025. Whole blood was collected aseptically from clinically suspected and apparently healthy animals during routine surveillance activities. Samples were transported to the laboratory by maintaining proper cold-chain conditions. Further, it was allowed to clot at room temperature and centrifuged at 3,000 xg/15 min for serum separation. The sera were aliquoted and stored at -20 °C until serological evaluation. The list of serum samples collected is mentioned in Table 1.

2.2 Protocol for ID Screen® African swine fever Competition ELISA kit

The following protocol was used for the screening of serum samples in ID Screen® African swine fever Competition ELISA kit. All the reagents were allowed to thaw at room temperature before use. All reagents were homogenized by inversion or vortexing. Firstly, 50 µL of dilution buffer 19 was added to each well of the microplate provided in the kit. Then, 50 µL of the positive control (PC) and negative control (NC) were added to the positive and negative control wells respectively, after which, serum samples were added, 50 µL each, to the remaining wells. The plate was covered and incubated for 45 min ± 4 min at 37 °C (± 3 °C). The wells were emptied and each well was washed 3 times with at least 300 µL of the wash solution. Drying of the wells was avoided between the washes. 1X conjugate was prepared by diluting the 10X conjugate to 1:10 in dilution buffer 12. 100 µL of the 1X conjugate was added to each well. The plate was covered and incubated for 30 min ± 3 min at 37 °C (± 3 °C). The wells were emptied and washed 3 times with at least 300 µL of the wash solution while avoiding drying of the wells between washes. 100 µL of the substrate solution was added to each well. The microplate was covered and incubated for 15 min ± 2 min at 21 °C (± 3 °C) in the dark. 100 µL of the stop solution was added to each well in the same order as in step (9) to stop the reaction. The optical density (OD) at 450 nm was read and recorded within 5 min of stopping the reaction.

2.3 Protocol for E-lab Science ASF Competition ELISA kit

The following protocol was used for the screening of serum samples in E-lab Science ASF Competition ELISA kit. All the reagents were allowed to come to room temperature ($21^{\circ}\text{C} \pm 5^{\circ}\text{C}$) before use. All reagents were homogenized by inversion or vortexing. Firstly, 50 μL of sample diluent was added to each well of the microplate provided in the kit. Then, 50 μL of the positive control (PC) and negative control (NC) were added to the positive and negative control wells respectively, after which, serum samples were added, 50 μL each, to the remaining wells. The plate was covered with a sealer and incubated for 60 min at $37^{\circ}\text{C} (\pm 3^{\circ}\text{C})$ in shading light. The wells were emptied and each well was washed 4 times with at least 300 μL of the wash buffer. Drying of the wells was avoided between the washes. Then, 50 μL of the HRP conjugate was added to each well. The plate was covered again with sealer and incubated for 30 min at $37^{\circ}\text{C} (\pm 3^{\circ}\text{C})$ in shading light. The wells were emptied and washed 4 times with at least 300 μL of the wash buffer while avoiding drying of the wells between washes. Lastly, 100 μL of the substrate reagent was added to each well and mix thoroughly. The microplate was covered and incubated at $37^{\circ}\text{C} (\pm 3^{\circ}\text{C})$ for 15 min in the dark. Followed by addition of 50 μL of the stop solution to each well in the same order as in previous step to stop the reaction. The optical density (OD) at 450 nm was read and recorded within 10 min of stopping the reaction.

2.4 Data analysis

The obtained data regarding the number of positive and negative samples in both the commercial ELISA kits (ID Screen® African swine fever Competition ELISA kit, France and E-lab Science ASF Competition ELISA kit, China) were compared to check the level of agreement between both the tests.

3. Results

3.1 Screening of serum samples

All serum samples ($n = 330$) were initially screened for the presence of ASFV-specific antibodies using two

commercially available ELISA kits (ID Screen® African swine fever Competition ELISA kit, France and E-lab Science ASF Competition ELISA kit, China). Based on the manufacturer's recommended cut-off criteria, samples were classified as positive or negative and subsequently used for further assessment. The test results showing overall seropositivity of ASF in Uttar Pradesh using ID Screen ASF kit and E-lab Science ASF kit have been calculated to be 11.8% and 10.9% respectively (Table 1) and illustrated in (Fig.1).

3.2 Results interpretation in ID Screen® African swine fever Competition ELISA kit

The test was validated if OD of negative control (NC)-greater than 0.7; ratio of positive control OD and negative control OD was less than 0.3. For each sample, the competition percentage (S/N%) was calculated using the given formula: $S/N\% = [(OD \text{ samples} - OD \text{ of positive control}) / (OD \text{ of negative control} - OD \text{ of positive control})] \times 100$. The samples presenting S/N%: less than or equal to 40% were considered positive, between 40% and 50% were considered doubtful and greater than or equal to 50% were considered negative.

3.3 Results interpretation in E-lab Science ASF Competition ELISA kit

The test was validated if OD of negative control (NC)-greater than 0.5; percentage inhibition (PI%) of positive control-greater than 60%. For positive control, the percentage inhibition (PI%) was calculated using the given formula: $PI\% = (1 - OD \text{ of positive control} / OD \text{ of negative control} \times 100\%)$. For each sample, the percentage inhibition (PI%) was calculated using the given formula: $PI\% = (1 - OD \text{ samples} / OD \text{ of negative control} \times 100\%)$. The samples presenting PI%: less than or equal to 50% were considered negative and greater than 50% were considered positive.

3.4 Level of agreement

The level of agreement of the test results in ID Screen® African swine fever Competition ELISA kit, France with E-lab Science ASF Competition ELISA kit, China was calculated to be greater than 90%.

Table 1: Detailed table of serum samples collection in several districts of Uttar Pradesh

S. No.	Place of collection	Year of collection	No. of samples	Status			
				ID Screen ELISA		E Lab Science ELISA	
				Positive	Negative	Positive	Negative
1	Gorakhpur	2025	102	13	89	12	90
2	Bareilly	2025	93	9	84	9	84
3	Azamgarh	2025	47	4	43	3	44
4	Mau	2025	30	6	24	6	24
5	Balia	2025	35	4	31	4	31
6	Pilibhit	2025	23	3	20	2	21
Total			330	39	291	36	294

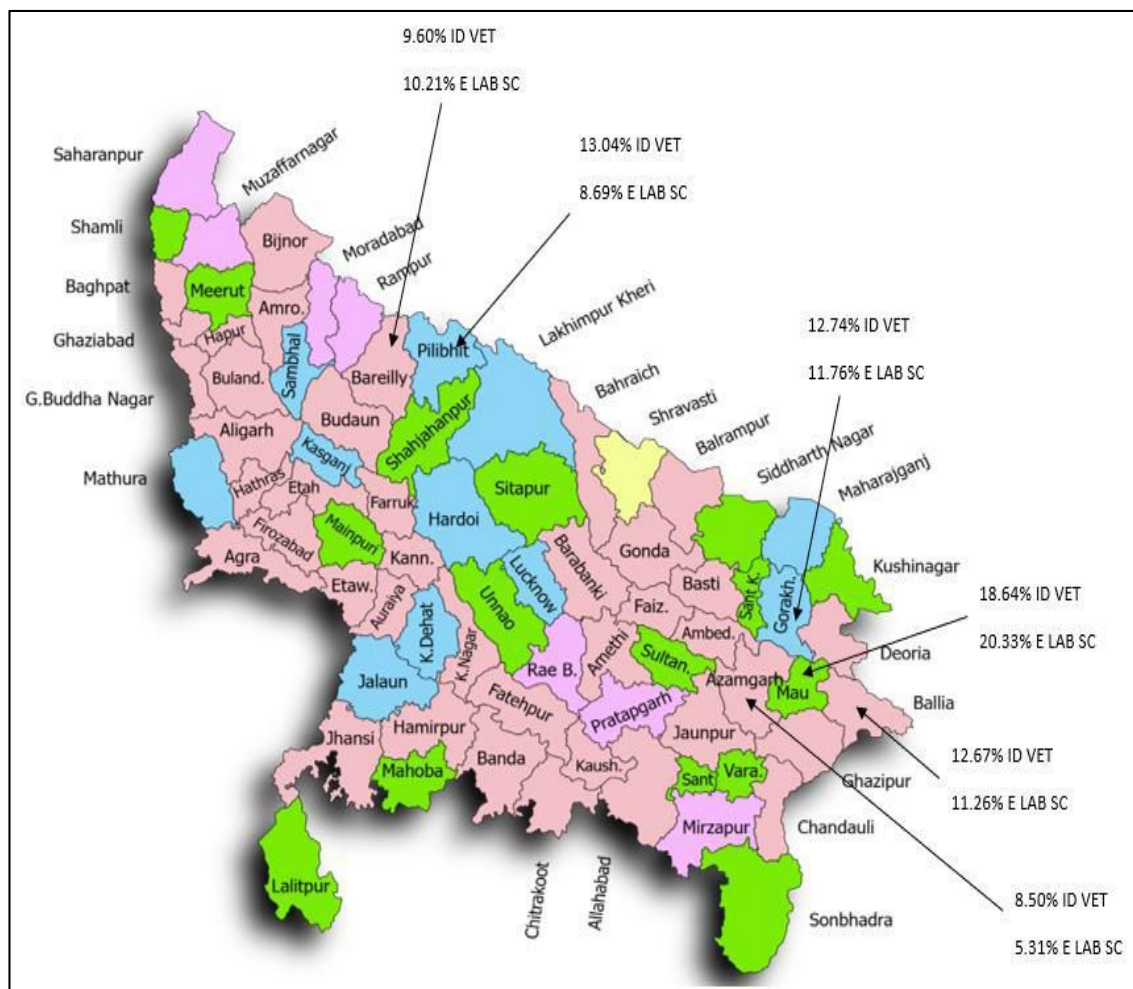


Fig 1: Sero-surveillance of ASF in different districts of Uttar Pradesh

4. Discussion

African swine fever (ASF) is a highly contagious viral disease affecting both domestic and wild pigs and has posed a profound and persistent threat to the global swine industry for over a century (Galindo and Alonso, 2017) [5]. In the absence of an approved vaccine and given the virus's pronounced capacity for transboundary dissemination, effective control and prevention strategies are critically dependent on timely and accurate diagnosis, coupled with stringent biosecurity practices (Penrith and Vosloo, 2009) [18]. Because vaccination is unavailable, serological detection of ASFV-specific antibodies remains a reliable marker of natural infection. Antibodies typically emerge within 7-10 days following infection and persist for extended periods, rendering serological assays indispensable tools for ASFV surveillance and diagnosis. Substantial evidence indicates that the identification and use of highly immunogenic viral proteins are vital to the advancement of sensitive and reliable serological diagnostic platforms (Yuan *et al.*, 2021) [25]. The use of recombinant antigens in ASFV sero-diagnostics offers distinct advantages, including the elimination of risks associated with handling infectious virus and the avoidance of high-containment (BSL-3) laboratory requirements. Owing to their operational simplicity, safety, and cost-effectiveness, serological assays represent one of the most practical approaches for detecting ASFV-specific antibodies.

In the absence of any other indigenous serological diagnostic assay/commercial ASF antibody detection ELISA kit in India. The best available option for the sero-

surveillance study was by using available ELISA commercial kits from reputed companies. The tested porcine serum samples comprised of samples collected from infected pigs and also the ones collected at random in the state of Uttar Pradesh. Highly specific nature of competition ELISA is due to the fact that virus-specific antibodies in samples react with antigen to block or compete with the binding of polyclonal antibody to the antigen. At present, no indigenously developed antibody detection ELISA assay is available in India where ASF is incessantly creating havoc in nearly 80% of Indian states. In this current study, we have screened the porcine serum samples using two commercial kits:-ID Screen® African swine fever Competition ELISA kit, France and E-lab Science ASF Competition ELISA kit, China. The coating antigen in the former kit is ASFV P30 whereas in the latter one it is ASFV P30 and P72. Such a screening test using validated commercial kits can aid in timely detection of antibodies against ASFV in pigs of India. This screening test represents a valuable addition to the existing molecular methods for providing rapid and economical results. Additionally, it can contribute meaningfully to ASF surveillance, control, and disease containment efforts. However, the current study was only a pilot study limited to only one Indian state due to certain constraints. Furthermore, this work needs to be done at a large-scale including samples from other Indian states. Considering the large pig population in the country, an indigenously developed kit will be required for economical surveillance.

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5. Ethical approval

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to.

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7. Conflict of Interest

The authors declared no potential conflicts of interest concerning research, authorship, and/or publication of this article.

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