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African swine fever virus (ASFV) in the one health approach

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African swine fever virus (ASFV) in the one health approach

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Editorial: African swine fever virus (ASFV) in the one health approach

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KEYWORDS

African swine fever, epidemiology, virus, economic impact, genetic complexity

Editorial on the Research Topic
African swine fever virus (ASFV) in the one health approach

African Swine Fever (ASF) remains a critical threat to pig populations globally. The disease, caused by the African Swine Fever Virus (ASFV), has devastating effects on animal health, the swine industry, and the livelihoods of millions who rely on pig farming. With no commercially available vaccine and a complex epidemiology involving both domestic pigs and wild suids, ASF continues to be a top priority for researchers, veterinarians, and animal health authorities worldwide.

This Research Topic compiles nine recent studies that deepen our understanding of ASFV, enhance diagnostics and control, and explore the broader implications of outbreaks across different sectors.

An often-overlooked aspect of animal disease outbreaks is their impact on the mental health of responders. In this context, Bakke et al. examine the psychological toll on veterinarians involved in ASF control in the Philippines. The authors highlight the urgent need for psychological support, including access to counseling and training in emotional resilience, within outbreak response frameworks. Their findings highlight the emotional strain associated with disease management and call for the inclusion of mental health support in veterinary response planning.

Several studies focus on improving ASFV diagnostics. Zhang et al. describe a novel triple protein-based ELISA that improves sensitivity in antibody detection. Hu et al. introduce a duplex fluorescent qPCR assay capable of distinguishing genotype I, II, and recombinant strains currently circulating in China. Complementing these laboratory advances, Li et al. conduct a retrospective field study evaluating optimal sampling strategies for ASF surveillance.

On the biosecurity front, Hemmink et al. explore environmental mitigation tools, reporting virus-inactivating properties of rosin-functionalized plastic surfaces. This finding opens the door to novel materials that may enhance on-farm biosecurity practices (Hemmink et al.).

Feed safety is another area of interest, particularly due to concerns about indirect virus transmission. Shurson et al. assess the persistence of an ASF-like surrogate algal virus in feed ingredients under storage and digestion conditions, offering insights into feed-related risks and mitigation strategies.

Surveillance systems also benefit from novel data sources. Hsu et al. demonstrate that Google Trends can act as an early warning tool for ASF outbreaks in Southeast Asia, suggesting a role for digital surveillance to complement conventional approaches.

Spatial analysis supports targeted control strategies. Ko et al. apply a statistical model to identify ASF risk clusters in Korea, providing valuable guidance for regional planning and response.

Finally, Alotaibi et al. offer a broad review of ASFV reservoirs, transmission pathways, and genomic characteristics. Their work highlights gaps in wildlife surveillance and emphasizes the need for international cooperation in monitoring and control (Alotaibi et al.).

Collective insights and the path forward

Together, the studies presented in this Research Topic underscore the complexity of ASF and the need for interdisciplinary approaches. Key themes that emerge include:

- Enhanced diagnostics: new tools such as improved ELISAs and genotype-specific qPCRs are helping detect infections more accurately and quickly, supporting early intervention.
- Field sampling strategies: optimizing surveillance practices improves diagnostic yield and guides outbreak response more effectively.
- Environmental biosecurity: innovations like virus-inactivating surfaces offer passive defense mechanisms in high-risk environments.
- Feed-related risks: understanding viral persistence in feed leads to improved biosecurity and transport practices.
- Digital technologies: online behavior analysis provides promising support for early outbreak detection, particularly in under-resourced areas.
- Spatial modeling: mapping high-risk areas enables data-driven targeting of interventions, improving the cost-effectiveness and success of control strategies.
- Veterinary mental health: the emotional burden on frontline responders needs greater attention in disease response planning.
- Ecological and genetic complexity: a deeper understanding of wildlife reservoirs and viral genomics is vital for developing long-term control strategies.

The global fight against ASF requires collaboration across disciplines—virology, epidemiology, animal welfare, psychology, data science, and policy. No single approach can solve this challenge. Success will depend on integrating scientific advances with practical tools, stakeholder engagement, and shared international responsibility.

Final remarks

The articles collected in this Research Topic offer valuable scientific contributions that reflect both the scale and diversity of efforts underway to combat ASF. The research demonstrates that while ASF remains a formidable opponent, progress is being made on multiple fronts. Each study adds a piece to the larger puzzle of ASF prevention, detection, control, and, eventually, eradication.

Author contributions

FD: Writing – original draft, Writing – review & editing.

Conflict of interest

The author declares 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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A duplex fluorescent quantitative PCR assay to distinguish the genotype I, II and I/II recombinant strains of African swine fever virus in China

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African swine fever (ASF) is a severe, hemorrhagic, and highly contagious disease caused by the African swine fever virus (ASFV) in both domestic pigs and wild boars. In China, ASFV has been present for over six years, with three genotypes of strains prevalent in field conditions: genotype I, genotype II, and genotype I/II recombinant strains. In order to differentiate among these three ASFV genotypes, a duplex fluorescent quantitative PCR method was established using specific probes and primers designed based on viral genes MGF_110-1L and O61R from ASFV strains reported in the GenBank database. Following optimization of reaction conditions, a duplex fluorescent quantitative PCR method was successfully developed. This method demonstrated no cross-reactivity with porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), porcine reproductive and respiratory syndrome virus (PRRSV), classic swine fever virus (CSFV), porcine pseudorabies virus (PRV), porcine circovirus 2 (PCV2), porcine circovirus 3 (PCV3), highlighting its specificity. Sensitivity analysis revealed that the limits of detection (LODs) of this method were 2.95×10^{-1} copies/μL for the MGF_110-1L gene and 2.95×10^{0} copies/μL for the O61R gene. The inter- and intra-group coefficients of variation were both <1%, indicating high reproducibility. In summary, the establishment of this duplex fluorescent quantitative PCR method not only addresses the identification of the ASFV recombinant strains but also allows for simultaneous identification of the three epidemic genotype strains.

KEYWORDS

duplex fluorescent quantitative PCR, genotype identification, ASFV genotype I, ASFV genotype II, ASFV genotype I/II recombinant strain

Introduction

ASF is an acute, hemorrhagic, highly contagious disease caused by ASFV in both domestic pigs and wild boars. Due to its exceptionally high fatality rate, it has been classified as a notifiable animal disease by the World Organisation for Animal Health (WOAH) (1). ASFV was initially identified and isolated in Kenya in 1921, and it was introduced into China

in 2018, with subsequent reports of ASF outbreaks in other Asian countries (2, 3). ASFV is a member of the family *Asfarviridae* and is the sole arthropod-borne DNA virus in the genus *Asfivirus*. Classification of ASFV into 24 genotypes is based on the C-terminal sequence of the p72 gene (4). At present, China exhibits a predominance of three primary genotypes including genotype I, genotype II, and genotype I/II recombinant strains. The genotype II strains, first detected in China in 2018, demonstrate significant genetic similarity to the Georgia strain and is characterized by its high virulence, resulting in a clinical fatality rate of up to 100% (3). The EP402R gene, a key virulence factor of genotype II strains, encodes the CD2v protein and is responsible for the hemadsorption (HAD)-positive phenotype (3, 5). In contrast, genotype I strains, characterized by a low virulence phenotype of ASFV lacking the HAD phenotype, were identified in field samples from China in 2021 (6). Genotype I strains primarily manifest as chronic clinical symptoms without causing pig mortality, which is attributed to the absence of the MGF_505/360 genes in their genome and impaired expression of the EP402R gene (6, 7). Genotype I/II recombinant strains were first reported in 2023, demonstrating stronger virulence and higher transmissibility compared to genotype I and II strains (8). The recombinant strains are with mosaic genomes composed of 56.5% Georgia07-like genotype II virus and 43.5% NH/P68-like genotype I virus (8). Notably, the genotype-determining gene B646L of the recombinant strain is from genotype I virus, whereas the EP402R gene encoding CD2v is from genotype II virus (8). There is a lack of effective vaccines or drugs for combating ASFV, necessitating a focus on early and precise detection of ASFV in clinical samples as the primary method of prevention. The widely utilized approaches for detection and identification of ASFV are RT-qPCR methods. Due to the presence of multiple genotype strains, the accurate identification of genotypes through qPCR facilitates an initial assessment of the virulence of the infecting strains, thereby enabling the development of targeted control strategies against ASF outbreaks. Various methods have been applied for genotyping of ASFV, including qPCR (9), recombinase polymerase amplification (RPA)/recombinase-aid amplification (RAA) (10, 11), loop-mediated isothermal amplification (LAMP) (12), clustered regularly interspaced short palindromic repeats (CRISPR) (13) etc., targeting viral genes such as B646L, EP402R, E183L, I177L, MGF505-7R, MGF505-2R, MGF360-12L, and MGF360-14L (14).

However, the emergence of a genotype I/II recombinant strain in China has highlighted the inadequacy of current methods for typing all three genotypes simultaneously. This study presents a dual-fluorescence quantitative PCR method that can identify genotype I, genotype II, and genotype I/II recombinant strains by analyzing their genetic sequence characteristics. This method can be applied in the development of early warning, control, and recovery strategies in ASF outbreaks.

Methods and materials

Primers and probes

Ten ASFV genome sequences were chosen from the NCBI GenBank database, comprising 3 genotype I/II recombinant strains, 4 genotype I strains, and 3 genotype II strains (Table 1). The conservation and variation of sequences between the genotype I/II recombinant strains and other prevalent strains were analyzed using DNAstar Megalign software, as depicted in Figure 1. Primers and probes were designed separately for the genotype I/II recombinant strain with genotype I and genotype II strains, based on conserved regions, utilizing Primer Premier 6 software (Figure 1 and Table 2). Primers of MGF_110-1L-F and MGF_110-1L-R were specifically designed to target conserved regions of ASFV genotype I strains and genotype I/II recombinant strains, with the probe of MGF_110-1L-P emitting FAM fluorescence. Similarly, primers of O61R-F and O61R-R were designed to amplify conserved regions of ASFV genotype II strains and genotype I/II recombinant strains, with the probe of O61R-P emitting VIC fluorescence. These primers and probes were synthesized by Sangon Biotech (Shanghai) Co., Ltd., diluted to a concentration of 10 μM with ddH_2O, and stored at −20°C.

Standard plasmid

The pUC57-ASFV standard plasmid was generated through the synthesis and cloning of gene sequences amplified from the MGF_110-1L gene of ASFV genotype I isolate (MZ945537) and the O61R gene of genotype II isolate (MK333180) in GenBank into the pUC57 vector. Quantification of the standard plasmid was performed

TABLE 1 Information of the reference strains.

Number	Strains	GenBank number	Year	Genotype
1	Pig_Henan_123014_2022	OQ504954	2022	I/II recombinantion
2	Pig_Inner Mongolia_DQDM_2022	OQ504955	2022	I/II recombinantion
3	Pig_Jiangsu_LG_2021	OQ504956	2021	I/II recombinantion
4	Pig_SD DY_2021	MZ945537	2021	I
5	PigHeN_ZZ-P1_2021	MZ945536	2021	I
6	OURT88_3	AM712240	1988	I
7	47_Ss_2008	KX354450	2008	I
8	ASFV_SY18	MH766894	2018	II
9	Pig_HLJ2018	MK333180	2018	II
10	Georgia_2007	FR682468	2007	II

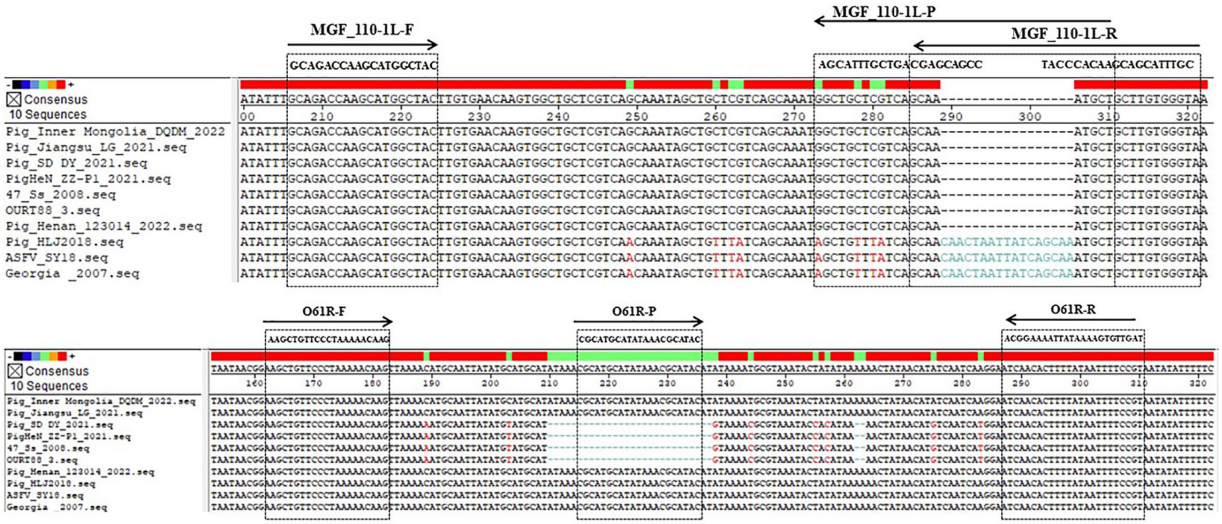

FIGURE 1
Location of primer and probe sequences in the genomes of different ASFV strains.

TABLE 2 Sequences of primers and TaqMan probes.

Name	Sequence (5′–3′)	Fluorescence signal	Product size (bp)
MGF_110-1L-F	GCAGACCAAGCATGGCTAC	—	138
MGF_110-1L-R	TACCCACAAGCAGCATTTGC	—	
MGF_110-1L-P	FAM-AGCATTTGCTGACGAGCAGCC-BHQ1	5′FAM-3′BHQ1	
O61R-F	AAGCTGTTCCCTAAAAACAAG	—	149
O61R-R	ACGGAAAATTATAAAAGTGTTGAT	—	
O61R-P	VIC-CGCATGCATATAAACGCATAC-BHQ1	5′VIC-3′BHQ1	

using a UV–visible spectrophotometer, and copy numbers were determined using a specific formula (15). Subsequently, a 10-fold serial dilution was conducted, resulting in concentrations ranging from 2.95×10^{9} to 2.95×10^{-1} copies/μL, which were then stored at −20°C for further use.

Optimization of reaction conditions

Concentrations of primers and probes were optimized by a matrix method. Various concentrations of primers (10 μM) ranging from 0.2 to 0.8 μL each, and probes (10 μM) ranging from 0.1 to 0.4 μL each, along with annealing temperatures between 55°C and 61°C, were tested to achieve the desired optimization. The objective was to minimize the Cq value and maximize the fluorescence intensity (RFU) in the reaction.

Evaluation of sensitivity and construction of standard curves

Standard plasmids of pUC57-ASFV were utilized as templates for the duplex fluorescent quantitative PCR method, with 10-fold serial dilutions ranging from 2.95×10^{9}–2.95×10^{-1} copies/μL. This method was employed for dual-fluorescence quantitative PCR amplification to generate an amplification kinetics curve and assess the sensitivity. The LODs of the MGF_110-1L gene and the O61R gene were evaluated by plotting the concentration of standard plasmids on the *x*-axis and the cycle threshold (Cq value) on the *y*-axis.

Evaluation of specificity

The duplex fluorescent quantitative PCR method was employed to utilize cDNA of PEDV, TGEV, PRRSV, CSFV, and DNA of PRV, PCV2, and PCV3 as templates. The pUC57-ASFV standard plasmid served as the positive control, while ddH_2O was utilized as the negative control to assess the specificity of this method.

Evaluation of reproducibility

Using standard plasmids of pUC57-ASFV with concentrations ranging from 2.95×10^{5} to 2.95×10^{1} copies/μL as templates, three batches of repeated tests were performed, with three replicates at each dilution within each batch. Cq values were statistically analyzed to calculate the intra- and inter-group coefficients of variation.

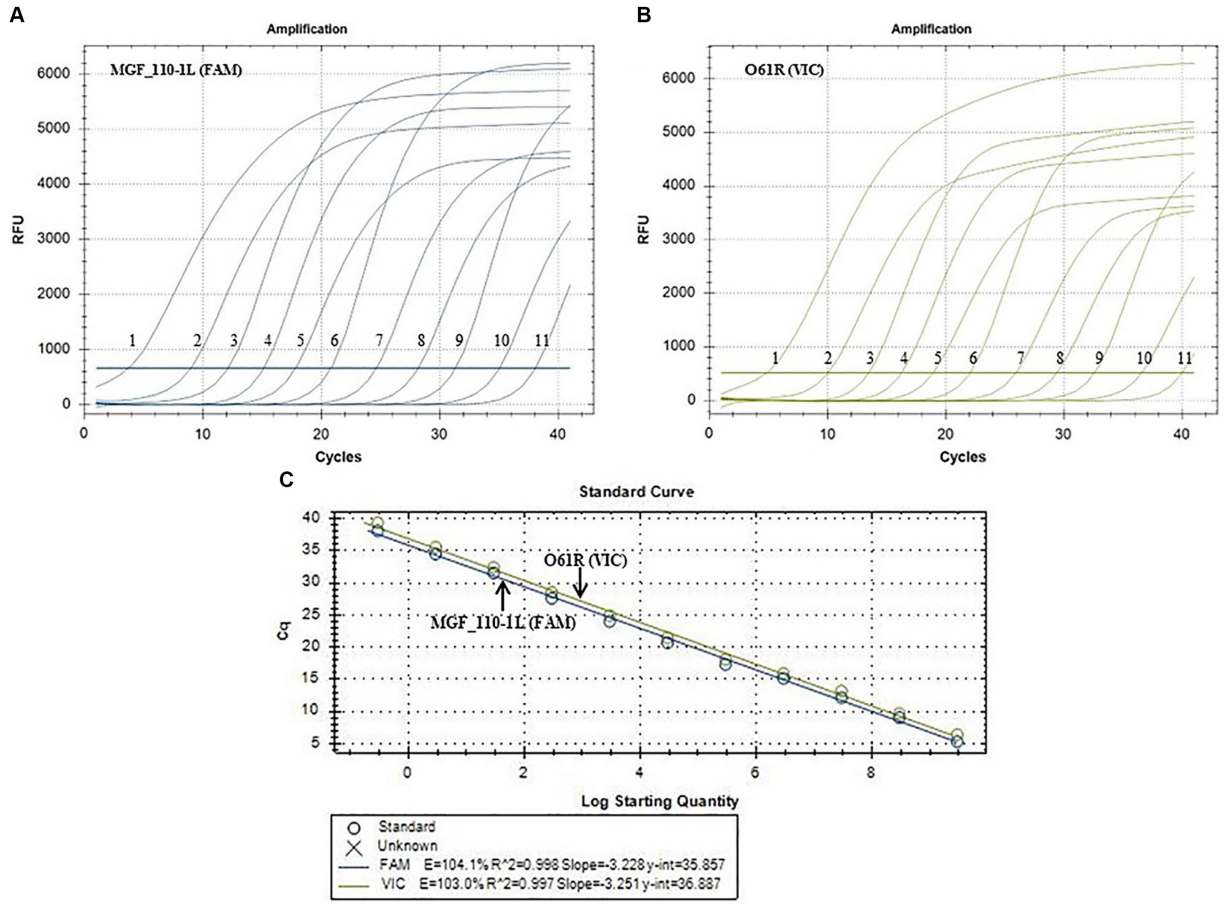

FIGURE 2
Sensitivity amplification curves and standard curves of the duplex fluorescent quantitative PCR. **(A,B)** The sensitivity amplification curves of MGF_110-1L gene with FAM channel **(A)** and O61R gene with VIC channel **(B)**. Number 1–11: 2.95×10^{9}–2.95×10^{-1} copies/uL. **(C)** Standard curves of both MGF_110-1L gene and O61R gene.

Clinical sample testing

A total of 96 clinical samples were collected by farmers from pig farms in Shandong and Hebei Province and sent to our lab for testing, comprising 32 serum samples, 51 throat swabs, and 13 environmental wipe samples. DNA extraction was performed on 300 µL of serum, throat swab eluent, or environmental wipe eluent using the NPA-96E Automatic nucleic acid extractors from Bioer Technology Co., Ltd. (Hangzhou, China). Subsequently, 5 µL of the extracted DNA underwent qPCR detection using both the developed duplex fluorescent quantitative PCR and the method recommended by WOAH. The pUC57-ASFV standard plasmid served as the positive control, while ddH_2O was utilized as the negative control. A Cq value of <40 was considered as a positive result.

Results

Optimization of reaction conditions

Reaction conditions were optimized by the matrix method. The optimized 20 µL reaction system was as follows: 10 µL Probe Mix, 0.2 µL each of upstream and downstream primers (10 µM), 0.1 µL probes (10 µM), 4 µL template, and ddH_2O added to a final volume of 20 µL. The reaction program was as follows: 37°C for 2 min; 95°C for 5 min; 95°C for 10 s, 60°C for 30 s, for 40 cycles.

Evaluation of sensitivity and construction of standard curves

Positive plasmids were utilized as templates for fluorescence quantitative PCR amplification following a 10-fold gradient dilution, resulting in a concentration range of 2.95×10^{9}–2.95×10^{-1} copies/µL. As shown in Figure 2, the LOD for the MGF_110-1L gene was 2.95×10^{-1} copies/µL (Figure 2A) and the O61R gene was 2.95×10^{0} copies/µL (Figure 2B), demonstrating the excellent sensitivity of the detection method established in this study. Furthermore, standard curves were automatically generated by the fluorescence quantitative PCR instrument. The standard curve for the MGF_110-1L gene exhibited a linear equation of $Y = -3.228X + 35.857$, with a coefficient of determination (R^2) of 0.998 and an efficiency (Eff%) of 104.1%. Similarly, the standard curve for the O61R gene showed a linear equation of

$Y = -3.251X + 36.887$, with an R^2 of 0.997 and an Eff% of 103.0%, as illustrated in Figure 2C. The aforementioned results demonstrated a strong linear correlation between the quantity of template and Cq value across the range of diluted concentrations.

Evaluation of specificity

The optimized reaction protocol was utilized for the detection of nucleic acids from various porcine viruses, including PEDV, TGEV, PRRSV, CSFV, PRV, PCV2 and PCV3. As shown in Figure 3, the result illustrated the absence of amplification curves for the aforementioned pathogens or the negative control, suggesting no cross-reactivity with common porcine viruses.

Evaluation of reproducibility

As shown in Table 3, the intra-group coefficients of variation ranged from 0.23 to 0.51%, and the inter-group coefficients of variation ranged from 0.12 to 0.4%. The results indicated the excellent reproducibility of this method.

Clinical sample testing

Results depicted in Figure 4 indicated that out of the 96 clinical samples, 17 tested positive for ASFV, which was consist with results by the WOAH method. Among these positive samples, 5 were identified as ASFV genotype I strains, 6 as ASFV genotype II strains, and 6 as ASFV genotype I/II recombinant strains. These findings suggested that the method developed in this study can effectively be utilized for the laboratory diagnosis and identification of ASFV genotypes.

Discussion

ASFV, a DNA virus, demonstrates notable levels of variability and frequency of variant strains. In China, three genotypes of ASFV have been identified: genotype I, genotype II, and genotype I/II recombinant strains (3, 6, 8). Additionally, the emergence of commercial gene-deleted vaccines, such as the ASFV-△I177L vaccine, has raised safety concerns (16–18) to the pig industry. Consequently, the genetic diversity of these viral strains has spurred researchers to continually enhance and innovate detection methods and technologies.

There have been some methods currently in use for identification of ASFV genotypes (14). Among these methods, qPCR is commonly favored by large-scale farms due to its high sensitivity and relatively low cost. Cao et al. (19) have devised a qPCR assay that specifically targets the B646L gene for distinguishing between ASFV genotype I and genotype II strains, achieving a LOD of 10 copies per reaction. It was observed that there was only a single base variation in PCR products between ASFV genotype I and genotype II strains in this method. However, recombinant strains are categorized as genotype I based on their B646L gene sequence (8), indicating that the B646L gene may not be a reliable target for distinguishing among the three ASFV genotypes. Additionally, Gao et al. (20) have developed a dual real-time PCR assay to differentiate between genotype I and genotype II by targeting two genes, B646L and E183L, with LODs of 1.07×10^2 copies/μL for B646L and 3.13×10^4 copies/μL for E183L. It was observed that there was only a single base disparity in the E183L gene in PCR products and no disparity in the B646L gene in PCR products between genotype I and genotype II strains, potentially constraining the sensitivity of this methodology. To concurrently identify the three genotypes and enhance sensitivity, a comparative analysis was performed in this study on the sequences of genotype I, genotype II, and genotype I/II recombinant strains to elucidate the variations and similarities among them. Subsequently,

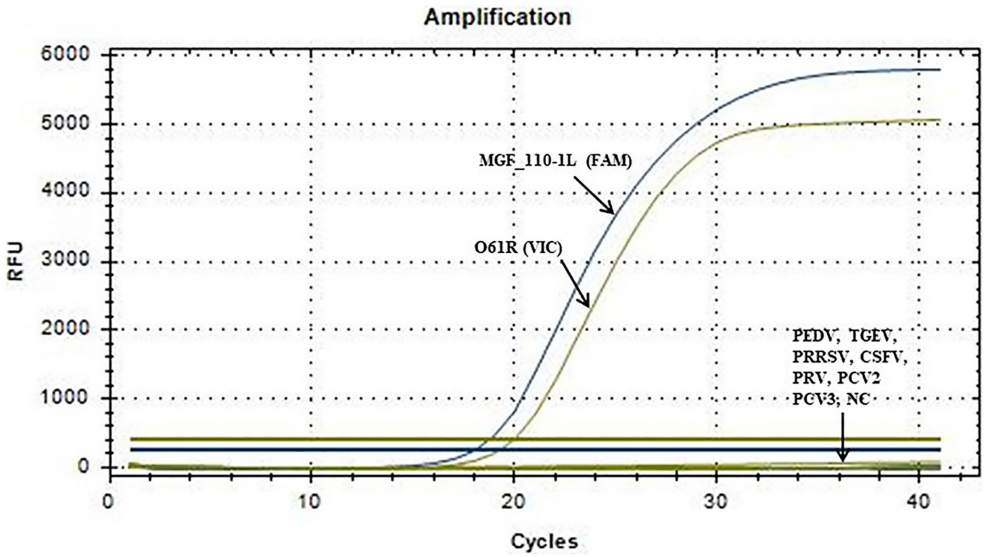

FIGURE 3
Specific amplification curve of the duplex fluorescent quantitative PCR. No detection signal was obtained for PEDV, TGEV, PRRSV, CSFV, PRV, PCV2, PCV3 or NC.

TABLE 3 Intra-reproducibility and intra-repeatability of the MGF_110-1L gene and O61R gene by the duplex fluorescent quantitative PCR.

Target gene	Template concentration (copies /μL)	Intra-assay variation			Intra-assay variation		
		Average value	Standard deviation	CV	Average value	Standard deviation	CV
MGF_110-1L	2.95×10^5	17.73	0.09	0.51%	17.91	0.07	0.39%
	2.95×10^4	20.74	0.05	0.24%	21.49	0.08	0.37%
	2.95×10^3	24.72	0.08	0.32%	24.63	0.05	0.20%
	2.95×10^2	28.06	0.13	0.46%	28.29	0.11	0.39%
	2.95×10^1	31.13	0.13	0.42%	31.66	0.09	0.28%
O61R	2.95×10^5	18.72	0.07	0.37%	18.66	0.06	0.32%
	2.95×10^4	21.89	0.09	0.41%	22.36	0.03	0.13%
	2.95×10^3	25.89	0.06	0.23%	25.63	0.03	0.12%
	2.95×10^2	29.21	0.08	0.27%	29.35	0.09	0.31%
	2.95×10^1	32.30	0.11	0.34%	32.82	0.13	0.40%

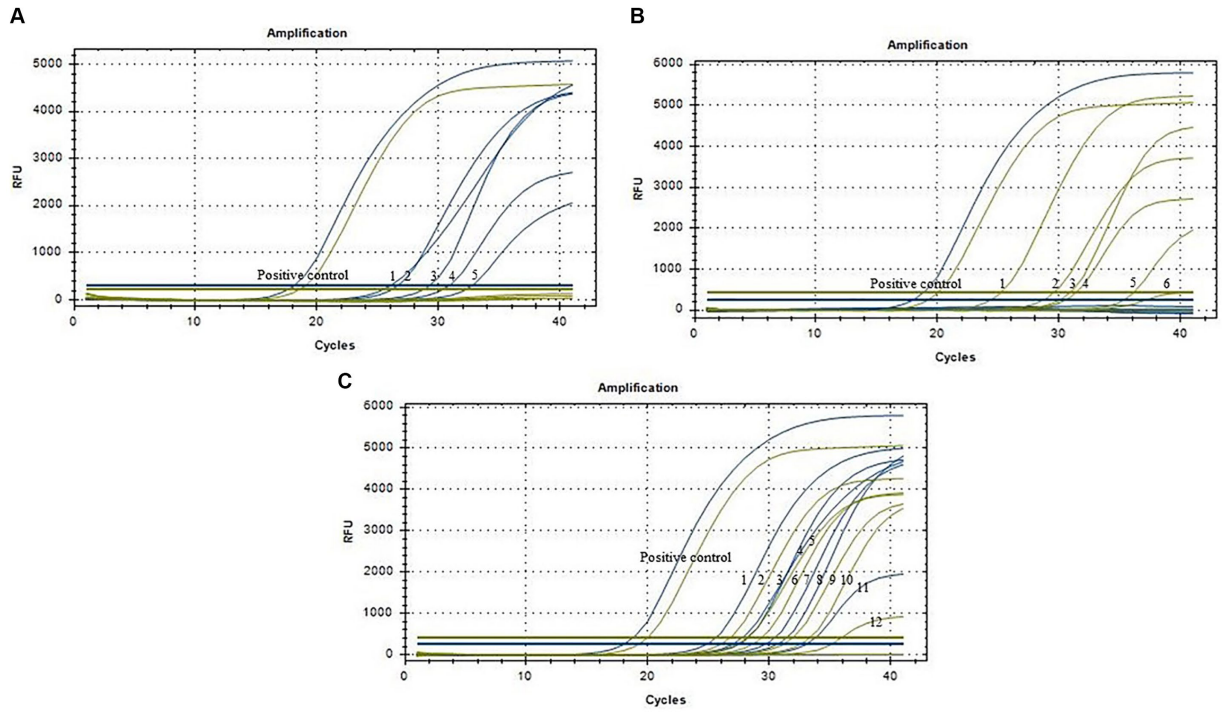

FIGURE 4
Amplification curves of clinical samples (*N* = 96). **(A)** Five positive samples of ASFV genotype I strains (No. 1–5). **(B)** Six positive samples of ASFV genotype II strains (No. 1–6). **(C)** Six positive samples of ASFV genotype I and II recombinant strains (No. 1–12). Blue lines represent the MGF_110-1L gene, and yellow lines represent the O61R gene.

the MGF_110-1L gene and the O61R gene were selected for additional scrutiny. There was a difference of 9 bases in the MGF_110-1L gene PCR products between genotype I or I/II recombinant strains and genotype II strains, and a difference of 21 bases in the O61R gene PCR products between genotype II or I/II recombinant strains and genotype I strains (Figure 1). The LODs for this method were 2.95×10^{-1} copies/μL for the MGF_110-1L gene and 2.95×10^{0} copies/μL for the O61R gene, which were lower than previously reported values (19, 20). Additionally, this method represents the latest approach capable of distinguishing among genotype I, genotype II, and genotype I/II recombinant strains concurrently. Furthermore, the target genes offered have potential applications in clinical detection for large-scale farms and could also contribute to the development of on-site detection methods when integrated with complementary techniques like LAMP, CRISPR and so on.

The methodology outlined in this study is primarily suited for two key applications. Firstly, it enables precise identification of positive samples obtained from pigs exhibiting suspected clinical symptoms, including throat swabs, blood, and tissue samples, facilitating accurate virus assessment by farmers and veterinarians in the early stages of infection. Additionally, ASFV can survive in the environment for

extended periods and can be transmitted through contaminated materials (1, 21). Therefore, precise identification of samples pertaining to the surroundings of swine farms can aid farmers and veterinarians in implementing specific biosecurity measures prior to the onset of infection on the farm, thereby averting the occurrence of ASFV.

In conclusion, the establishment of this duplex fluorescent quantitative PCR method not only addresses the deficiency in identifying recombinant strains but also allows for the simultaneous identification of the three genotypes. This provides a theoretical basis for the formulation of targeted prevention and control strategies against ASFV outbreaks.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study uses samples obtained from commercial pig farms. Dezhou Animal Disease Prevention and Control Center did not require the study to be reviewed or approved by an ethics committee because the clinical samples used were provided by farmers from pig farms for ASFV diagnostic detection. Written informed consent was obtained from the owners of the animals to use the clinical samples.

Author contributions

ZH: Visualization, Formal analysis, Writing – review & editing, Writing – original draft, Conceptualization. RL: Resources, Methodology, Data curation, Writing – review & editing, Writing – original draft. XT: Writing – review & editing, Software, Resources, Methodology, Data curation. RG: Writing – review & editing, Software, Resources, Methodology, Data curation. XL: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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Conflict of interest

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Google trends as an early indicator of African swine fever outbreaks in Southeast Asia

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African Swine Fever (ASF) is a reportable disease of swine that causes far-reaching losses to affected countries and regions. Early detection is critically important to contain and mitigate the impact of ASF outbreaks, for which timely available data is essential. This research examines the potential use of Google Trends data as an early indicator of ASF outbreaks in Southeast Asia, focusing on the three largest swine producing countries, namely, Vietnam, the Philippines, and Thailand. Cross-correlation and Kullback–Leibler (KL) divergence indicators were used to evaluate the association between Google search trends and the number of ASF outbreaks reported. Our analysis indicate strong and moderate correlations between Google search trends and number of ASF outbreaks reported in Vietnam and the Philippines, respectively. In contrast, Thailand, the country of this group in which outbreaks were reported last, exhibits the weakest correlation (KL = 2.64), highlighting variations in public awareness and disease dynamics. These findings suggest that Google search trends are valuable for early detection of ASF. As the disease becomes endemic, integrating trends with other epidemiological data may support the design and implementation of surveillance strategies for transboundary animal diseases in Southeast Asia.

KEYWORDS

African swine fever, Google trends, Vietnam, the Philippines, Thailand, public health, surveillance, epidemiology

1 Introduction

African Swine Fever (ASF), a highly contagious viral disease affecting pigs and wild boar, has had significant impacts on pig populations and the swine farming economy globally (1). Although the ASF virus does not infect humans, the disease poses a serious problem for food security worldwide. Following the introduction of ASF into China in August 2018, the disease spread through the Southeast Asia region and by December 2023, 19 countries in Asia had reported cases of ASF in either domestic pigs or wild boars (2). ASF disrupted the swine industry of South East Asia and the disease was first reported in the top three swine-producing countries in the region (Supplementary Table S1), Vietnam, the Philippines, and Thailand, in February 2019, July 2019, and January 2022, respectively.

A prerequisite for the assessment of the effectiveness of control measures for infectious diseases, such as ASF, is the availability of timely data on disease spread. However, those data are not always collected or reported in time due to a variety of factors, including resource limitations, data infrastructure, field conditions and reporting systems (3, 4). Alternatively, it has been proposed that the results of web searches may be used as a proxy for monitoring

disease presence or spread (5, 6). For example, Google Trends has been effectively used to forecast seasonal influenza outbreaks caused by Influenza Virus A in the United States (7) and various other countries, demonstrating a strong correlation and leading to proposals for an Internet surveillance system (8). Despite encountering limitations and complexities (9), Google Trends remains a freely accessible and user-friendly interface for epidemiological research on infectious diseases. Use of results of web searches as a proxy for disease spread may have an impact and application on South East Asia, a region that by 2021, had a population of 589 million individuals, with approximately 440 million (~75%) of them being active internet users. Vietnam, the Philippines, and Thailand have experienced a substantial increase in their online user base and digital consumer population. This surge can be attributed to the ongoing improvement of internet accessibility and infrastructure in the area, contributing to the sustained growth of online engagement and activity.

In this study, we aimed to compare the results of Google Trends with data on ASF spread in the top three swine producing countries of South East Asia (Thailand, Vietnam, and the Philippines). Because ASF was introduced into the region as an emerging disease, the hypothesis here is that online search volume may have a relation with the number of outbreaks within a country or a specific region. During the initial introduction of the disease into a country we expected a corresponding increase in Google searches as people seek information about the situation. Considering that ASF impacts not only swine farmers but also significantly affects pork prices and the swine supply chain, we expect a concurrent rise in search activity during severe ASF outbreaks.

The results presented in this study will help to understand the history of ASF spread in the region. Results also provide evidence for the use of the methodology to the monitoring of disease spread, which may have broader applications for transboundary animal disease surveillance in the region and globally, contributing to the ultimate goal of mitigating the impact of emerging animal diseases worldwide.

2 Materials and methods

2.1 Outbreak data collection and processing

ASF outbreak data from the Philippines, Vietnam, and Thailand was obtained from the Food and Agriculture Organization's EMPRES Global Animal Disease Information System (EMPRES-i).[1] Specifically, a total of 52 weeks of outbreak data subsequent to the first reporting of the disease on each country were retrieved and extracted from the system.

2.2 Google trend data

For secondary data or predictor, Google Trends[2] was sourced in this research. Google Trends distinguishes between search terms and search topics. Search terms are specific queries and their relative search volume within a given language, while search topics encompass a broader range of related terms irrespective of language. In our study, we adopted a "search topic selection" approach, integrating local languages to compare search patterns across different regions.

Google Trends offers insights into the volume of searches for particular keywords, providing an indication of the attention given to those topics in various countries. Concerning the African Swine Fever outbreak, keywords such as "African Swine Fever," "African Swine Fever Virus," and related terms were analyzed in local languages. Search topics were examined in both Vietnamese and English in Vietnam, in Thai and English in Thailand, and exclusively in English in the Philippines. Commonly used keywords revolved around "African Swine Fever" or "African Swine Fever Virus," with some users employing suggested terms such as "Swine Flu" or "*dịch tả lợn châu phi*" (Vietnamese for African Swine Fever). This reflects the varied search behaviors within each country. Keywords exhibiting significant peaks in search volume were selected for further investigation, while those with inadequate data were disregarded.

The trend data collection timeframe was tailored according to the date of each country's first officially confirmed case of ASF from EMPRES-i data. A period of 52 weeks before and after the initial detection was chosen to capture trends over time effectively. We analyzed up to three keywords, with the data normalized relative to the highest point of interest on the chart for the respective region and time frame. A score of 100 denotes peak popularity, while a score of 0 indicates insufficient data.

2.3 Statistical analysis

The ASF outbreak data and Google Trends data were aggregated on a weekly basis and juxtaposed for comparative analysis. To ensure synchronization of the timeline, date format was standardized according to the ISO week numbering system. Cross-correlation analysis was employed to measure the association between two signals or datasets—in this case, between Google Trends data and weekly African Swine Fever outbreak reports. This analysis helps to identify how the changes in one signal correspond to those in the other as they shift in time. In the context of our study, which focuses on the relationship between two time series (X and Y), it is posited that the Y series may be influenced by past time points of the X series. The sample cross-correlation function was utilized to identify lagged values of the X variable that could potentially predict changes in Y. Each lag represents a one-week interval in the results.

Kullback–Leibler (KL) divergence is a statistical measure for quantifying the difference between an arbitrary probability distribution and a reference probability distribution (10, 11). When interpreting the KL divergence value, a result of 0 indicates that the two distributions in question are identical. Conversely, an increase in divergence signifies that the arbitrary probability distribution deviates more from the reference distribution. Therefore, smaller KL divergence values are desired when the goal is to approximate the reference probability distribution closely with a predictive distribution.

The statistical analyses were conducted using R version 4.2.2. The similarity and possible time shifts between datasets were assessed with the ccf() function, which performs cross-correlation analysis. The KL divergence was calculated using the scikit-learn library, and the data was visualized by Python's Matplotlib.

1 https://empres-i.apps.fao.org/

2 https://trends.google.com/trends/

3 Results

3.1 ASF outbreak and Google trend correlation in Southeast Asia

In both Vietnam and the Philippines, a significant alignment was found between the epidemic curve and Google trends results (Figure 1). In Vietnam, the ASF outbreak began in Week 5 of 2019 (February) and peaked in Week 10. A secondary peak occurred during Weeks 19 and 20 (May 2019). In the Philippines, the first ASF outbreak was recorded in Week 29 (July 2019), reaching its highest point in Week 40 (October 2019). However, in the descriptive analysis for Thailand, before the initial detection of ASF, the Google trend curve showed numerous smaller peaks. This pattern contrasts with the trends observed in Vietnam and the Philippines.

3.2 Cross-correlation and KL divergence results in Southeast Asia

The cross-correlation analysis (Figure 2) provided insights into the time-lagged relationships between Google Trends search data and ASF outbreak in Southeast Asia. It highlighted the correlation peaks for different lags, suggesting patterns of synchronicity or shifts between search behavior and disease outbreaks. KL Divergence analysis (Figure 3) offered a quantifiable measure of the congruence between these two datasets.

Vietnam demonstrated the strongest correspondence between Google search trends and ASF outbreaks (KL = 0.54), indicating a close alignment between search behavior and outbreak patterns. The Philippines showed a similar pattern with a notable correlation (KL = 0.6). In contrast, Thailand exhibited the least correlation (KL = 2.64), suggesting its search behavior is less aligned with outbreak occurrences. These results reveal differences in search behavior and ASF outbreak trends across Southeast Asia.

4 Discussion

This research is the first to connect ASF outbreaks with Google Trends data, demonstrating that this tool is accessible and reproducible for analysis across various regions and languages in Southeast Asia. The results of the cross-correlation and quantifiable KL divergence analyses suggest that Google Trends can offer meaningful insights into patterns of public concern related to ASF.

Our analysis reveals varying levels of performance disparities among Vietnam, the Philippines, and Thailand. Vietnam demonstrates the most robust performance, with a lag of 0 and the lowest KL divergence, indicating a high degree of synchronization between the two datasets. Vietnam experienced the first ASF outbreak in Southeast Asia in February 2019, and as a new transboundary animal disease, public awareness of ASF was initially limited. This lack of awareness may have played a role in establishing a direct or indirect correlation between the severity of the outbreak and Google search volume. A similar pattern emerged in the Philippines, which also showed a low KL divergence value, with the ASF outbreak beginning in July 2019, about six months after the initial outbreak in Vietnam. This parallel timing suggests a comparable trend in public awareness and Google search activity.

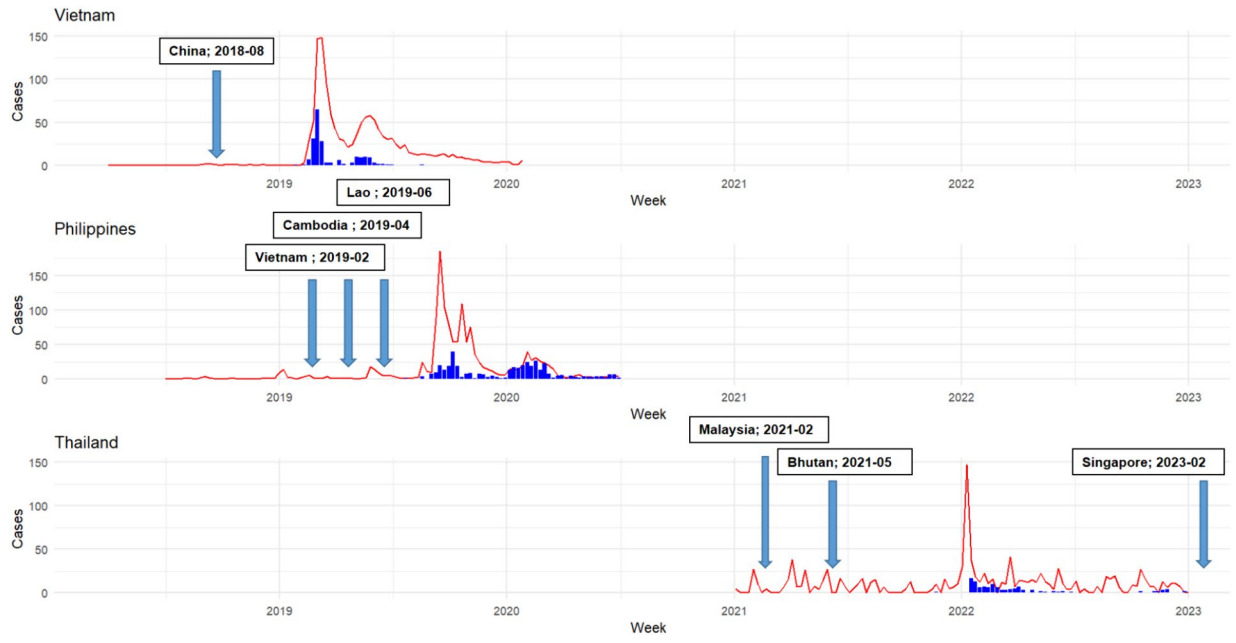

FIGURE 1
African Swine Fever (ASF) outbreaks (shown as blue bars) and Google Trends search volume for selected ASF-related keywords (represented by red lines) from 2018 to 2023 in Vietnam, Philippines, and Thailand. The arrows indicate when neighboring countries first reported ASF to the World Organization for Animal Health (WOAH). In the case of Vietnam and the Philippines, the timing of peaks in Google search volume (red line) aligns with peaks in ASF outbreak cases (blue bars), suggesting a synchrony between online search activity and the occurrence of ASF outbreaks in the field.

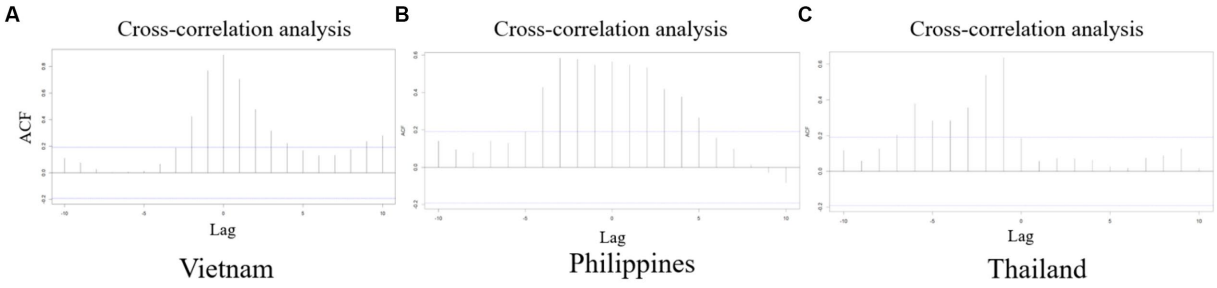

FIGURE 2
This figure presents the cross-correlation analysis between Google Trends search data and disease outbreaks. In the cross-correlation plot for Vietnam **(A)**, a significant correlation is indicated by peaks that exceed the blue dashed threshold line. A lag of 0 suggests a simultaneous peak in search data and ASF outbreaks, indicating a meaningful positive correlation. Additional peaks at lag −2 and lag 2 also indicate significant correlations. In the Philippines **(B)**, significant correlations are identified at lag −4 and lag 5, with the most prominent peak at lag −3. These peaks exceed the blue dashed threshold line, indicating a strong connection between Google Trends data and ASF outbreak timing. For Thailand **(C)**, no significant correlation is found at lag 0. However, there is notable correlation between lag −1 and lag −6, with the strongest peak at lag −1, suggesting a possible time shift between search trends and disease outbreaks.

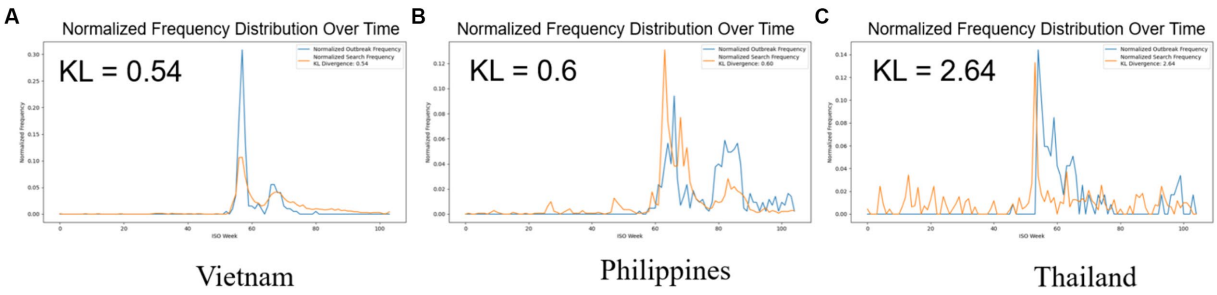

FIGURE 3
This figure uses KL Divergence to compare normalized ASF outbreak and Google search frequencies across three Southeast Asian countries. The blue lines represent ASF outbreaks, while the orange lines represent Google search frequencies. It displays the KL divergence values of the normalized search frequency with the reference to the normalized search frequency, with respective. The KL divergence quantifies the discrepancy between the two distributions, where a lower value indicates higher similarity. Results are as follows: **(A)** Vietnam demonstrates the closest alignment (KL divergence: 0.54), **(B)** Philippines shows moderate congruence (KL divergence: 0.6), and **(C)** Thailand exhibits the largest divergence (2.64), indicating less correlation.

However, as the ASF outbreak rapidly spread to Thailand and other Southeast Asian countries, entering its third year in the region, the dynamics shifted. Regional familiarity with the disease likely escalated, leading to a nuanced understanding of its awareness. This evolving awareness may elucidate the presence of peak noise in Google Trend results depicted in Figure 2 for Thailand, with an interval of 1 to 6 weeks difference.

ASF outbreaks in Vietnam and the Philippines were believed to have been mainly linked to the importation of pork products or tourists carrying items containing the genotype II ASF virus (12, 13). However, the transmission route in Thailand may have been different, likely related to owners importing pigs as companion animals. These imported pigs were later diagnosed with ASFV (14). On December 2021, the pig owner sent the carcass of one of the pigs to Kasetsart University for investigation into the sudden death. Subsequently, in January 2022, Thai authorities officially confirmed the presence of ASFV in the country, prompting the initiation of active and passive surveillance measures comprehensively (14). Currently, the spreading investigation from companion pigs to domestic farms and the transmission mechanism during the early stages of the ASF epidemic in Thailand remain unclear. There is also suspicion that the virus may have emerged earlier than indicated by official reports from Thailand authorities.

Our analysis of Thailand revealed a notable trend in Google searches: multiple search surges occurred before the official confirmation of the ASF outbreak in January 2022, indicating a potentially earlier occurrence than reported. While this observation alone may not be decisive, it raises questions about the timing of the first outbreak. Further evidence from Taiwanese airport surveillance showed that PCR testing detected ASFV in pork products from Nakhon Pathom province, Thailand, as early as September 30, 2021 (15). This molecular evidence strongly implied the possible infiltration of ASFV into Thailand's pork supply chain before official confirmation through local swine farm sampling. Integration of Google Trends methodology could potentially offer valuable insights into improving early detection efforts.

Using Google Trends to monitor disease outbreaks has its benefits and limitations. It can be a useful tool for gauging public concern in real-time during the early stages of an outbreak. Our analysis indicates that cross-correlation with Google search data works best when a transboundary animal disease is new to a

specific region, providing an effective predictive or indicative index. However, as a disease like ASF becomes endemic, the effectiveness of this approach may decrease, as the heightened public interest wanes over time. This underscores the need for flexible approaches when using search data for epidemiological surveillance and suggests that other strategies should be used alongside Google Trends to sustain relevance throughout the different phases of disease outbreak.

Several limitations of using Google Trends for disease outbreak analysis need to be considered. Although our methodology aims to reduce translation issues, direct translations can lead to a loss of subtlety. User search habits and behavior can vary, affecting search volume, and external factors such as news events, government policies, or marketing campaigns can influence public search patterns. Disparities in internet access and digital literacy can also introduce biases, potentially amplifying noise in urban areas and diminishing the signal in rural regions. Given these limitations, a surge in search activity might alert epidemiologists, but the data must be contextualized with other epidemiological information for a comprehensive understanding of ASF dynamics.

Lastly, our research is inherently biased by focusing on Google Search as the primary search engine in Southeast Asia. Exploring other search engines or locally popular social media platforms could offer additional insights. Studies have shown that platforms like Twitter can offer better precision in tracking diseases like the influenza virus through mathematical models in Greece (16). Therefore, exploring alternative competitive platforms or locally commonly used social media could enhance the effectiveness of disease outbreak analysis for future research directions.

In conclusion, our research demonstrates that comparing Google Trends data with official ASF outbreak data can reveal patterns of public interest that correspond to ASF outbreaks. This methodology, especially in a new region with emerging transboundary diseases, showed a significant correlation, suggesting that Google Trends could potentially serve as an early indicator for ASF outbreaks. However, to gain a comprehensive understanding of ASF dynamics, Google Trends data should be used in conjunction with other epidemiological information and interpreted with caution. This study highlights the value of flexible monitoring approaches and the need for further research into factors affecting public awareness and ASF outbreaks.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

C-HH: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft, Writing – review & editing. C-HY: Investigation, Methodology, Software, Visualization, Writing – review & editing. AP: Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing.

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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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Supplementary material

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A suitable sampling strategy for the detection of African swine fever virus in living and deceased pigs in the field: a retrospective study

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African swine fever (ASF) is a fatal disease that threatens the health status of the swine population and thus can impact the economic outcome of the global pig industry. Monitoring the ASF virus (ASFV) is of utmost concern to prevent and control its distribution. This study aims to identify a suitable sampling strategy for ASFV detection in living and deceased pigs under field conditions. A range of samples, comprising tissues obtained from deceased pigs, as well as serum and tonsil swab samples from live pigs, were gathered and subjected to detection using the qPCR method. The findings revealed that the mandibular lymph nodes demonstrated the highest viral loads among superficial tissues, thereby indicating their potential suitability for detecting ASFV in deceased pigs. Additionally, the correlations between virus loads in various tissues have demonstrated that tonsil swab samples are a viable specimen for monitoring live pigs, given the strong associations observed with other tissues. These findings indicated two dependable sample types for the detection of ASFV: mandibular lymph nodes for deceased pigs and tonsil swabs for live pigs, which supply some references for the development of efficacious preventive measures against ASFV.

KEYWORDS

African swine fever, mandibular lymph nodes, tonsil, qPCR, deceased and live pigs

Background

African swine fever (ASF) is a contagious disease caused by the African swine fever virus (ASFV), capable of infecting diverse porcine species. ASFV, belonging to the *Asfivirus* genus within the *Asfarviridae* family, is an enveloped double-stranded DNA virus with a diameter of approximately 200 nm, and is also the only DNA arthropod-borne virus discovered to date (1, 2). The first description of ASFV was done in Kenya in 1921, and then introduced from Africa to Portugal occurred in 1957, subsequently leading to outbreaks in various European countries (3–5). Notably, in 2018, China experienced its first outbreak in Shenyang City, which rapidly spread throughout the nation, posing a grave threat to the domestic pig industry (6). The transmission and excretion patterns of ASFV strains with different virulence were different

in domestic pigs. The advent of attenuated strains of ASFV has resulted in a gradual reduction of clinical symptoms, leading to significantly reduced viral loads in blood, saliva, and feces, and in some cases, the virus is completely undetectable by existing testing methods in these above sample types (7–9). Consequently, monitoring clinical manifestations becomes increasingly challenging.

Research has demonstrated that highly virulent ASFV strains typically require an incubation period in pigs for 9–12 days before they can be detected in the blood, whereas less virulent strains exhibit lower viral loads and intermittent clearance upon detection (10, 11). Moreover, the duration required for virus detection in the bloodstream varies depending on the various modes of infection, with contact transmission exhibiting a delay of 1–2 days compared to intramuscular injection (8, 11, 12). Additionally, ASFV has been reported to be able to spread through multiple routes, including contact and aerosol transmission (11, 13, 14). And also, ASFV-positive pigs have been found to harbor a significant quantity of virus particles in their blood and deep tissues (10, 11, 15–17), which indicates that the process of blood sampling and necropsy procedures may lead to extensive contamination of the virus within the facility in the event of ASFV outbreaks in large-scale farms. Consequently, the accurate evaluation of ASFV presence in appropriate clinical samples is crucial for mitigating ASFV transmission in extensive commercial pig operations (18). This study aims to compare viral loads across various tissues, thereby establishing a benchmark for ASFV detection and early warning systems.

Materials and methods

Sample source

All the samples consisted of clinical disease materials that had been accumulated and preserved at a temperature of −80°C in our laboratory over the past 3 years. The tissue samples were obtained from 23 suspected dead-ASFV positive pigs, which were collected by trained veterinarians and subsequently sent to our laboratory for qPCR testing within 24 h of sample collection. Each tissue sample of 0.02 g was subjected to grinding using a tissue grinder, and subsequently mixed with 1 mL of PBS. Serum samples ($N = 74$) and tonsil swab samples ($N = 74$) were acquired from live pigs by trained veterinarians in several ASFV-positive herds undergoing ASF precision culling, and sent to our laboratory for testing within 24 h of sample collection. In case of serum collection, 5 mL of blood was extracted from the anterior vena cava of each pig, from which 1–2 mL of serum was isolated. Tonsil swab sampling involved the insertion of swabs into the deep tonsil position of pigs, which were then held for a minimum of 3 s, removed, and subsequently dissolved in 3 mL of normal saline.

qPCR

All the samples were tested using qPCR following the previously described method (19). Briefly, 300 μL of serum or throat swab eluent were subjected to DNA extraction using the Automatic nucleic acid extractors (NPA-96E) from Bioer Technology Co., Ltd. (Hangzhou, China). Subsequently, 5 μL of the extracted DNA was utilized for qPCR detection, which was performed on a Step One Plus instrument (ABI) using the PerfectStart® II Probe qPCR SuperMix (TransGen Biotech, China) according to the manufacturer's instructions. Specific primers for the ASFV B646L gene were designed based on the ASFV isolate Pig/HLJ/18 (GenBank: MK333180.1) (18) and used for qPCR: 5'-AAAATGATACGCAGCGAAC-3'(forward), 5'-TTGTTTACC AGCTGTTTGGAT-3' (reverse), and 5'-FAM-TTCACAGCATTT TCCCGAGAACT-BHQ1-3' (probe) (17). The results of qPCR were recorded as quantification cycle values (Cq values), and a Cq value of <40 was considered as a positive result. The limit of detection (LOD) of this method is 2.5 copies/μL (19), which is more sensitive than the method recommended by World Organization for Animal Health (WOAH) (20).

Statistical analyses

The mean copy numbers among different tissues were compared using the paired t-test in the GraphPad Prism software (version 8.0). The Pearson correlation analysis between different tissues was performed to construct the correlation coefficient matrix and the relation coefficient between serum and tonsil swab samples using the GraphPad Prism software (version 8.0) as well. A p value of <0.05 was considered to be statistically significant.

Results

Viral loads in different tissues

To identify the distribution of the virus in different tissues of deceased pigs, a comparison of viral loads in various tissues from the same pig were conducted. As shown in Figure 1, there was a descending order of viral loads from high to low: lung, spleen, mediastinal lymph nodes, mandibular lymph nodes, bronchial lymph nodes, tonsils, and inguinal lymph nodes. Notably, in superficial tissues, the viral loads in mandibular lymph nodes and tonsils were

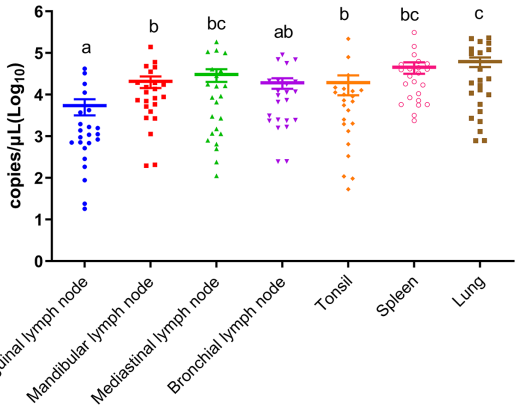

FIGURE 1
Viral loads of ASFV in inguinal lymph nodes, mandibular lymph nodes, mediastinal lymph nodes, bronchial lymph nodes, tonsils, spleens, and lungs. Different letters indicate significant statistical differences ($p < 0.05$), and the same letter indicates no significant statistical differences ($p > 0.05$).

significantly higher than those in inguinal lymph nodes ($p < 0.05$), suggesting that mandibular lymph nodes are a viable option for detecting ASFV under field conditions.

Correlation analysis between different tissues

Through the examination of virus load correlations across various tissues, as shown in Figure 2A, the investigation revealed that the most substantial correlation ($R = 0.91$, $p < 0.001$) was observed between tonsils and mandibular lymph nodes, followed by the correlations between tonsils and bronchial lymph nodes ($R = 0.61$, $p < 0.05$). Furthermore, a significant correlation was also identified between tonsils and mediastinal lymph nodes ($R = 0.51$, $p < 0.05$). These findings suggest that tonsils may serve as a suitable choice for sample collection in pigs. Moreover, a total of 74 positive pigs were subjected to the collection of serum and tonsil swab samples, and their correlation was subsequently analyzed. The findings shown in Figure 2B demonstrate a correlation coefficient (R value) of 0.74 between tonsil swabs and serum samples, suggesting that tonsil swabs possess the potential to serve as a viable alternative to serum samples for the detection of ASFV in live pigs.

Discussion

ASF is a profoundly deleterious ailment, exhibiting an almost absolute fatality rate. Its clinical progression encompasses hyper-acute, acute, sub-acute, and chronic phases (21). The incubation period of ASFV varies, contingent upon the distinct strains, modes of infection, and the immunological status of the host pig, typically spanning from 3 to 19 days (22–24). Pigs infected with highly virulent or moderately virulent strains exhibit an elevated mortality rate, and the virus disseminates within the porcine population even prior to the manifestation of clinical symptoms (11). Hence, it is crucial to promptly detect and ascertain the presence of ASFV infection in pig populations to effectively manage and mitigate the losses incurred by ASFV outbreaks (25).

Presently, ASFV surveillance is predominantly carried out using two approaches: passive and active monitoring. Passive monitoring primarily entails the surveillance of deceased pigs. Sample types for ASFV detection in deceased pigs, as recommended by WOAH, include spleen, lymph nodes, bone marrow, lung, tonsil and kidney (16, 26). Clinical sample types that have been successful in isolating ASFV strains primarily consist of the spleen, lymph nodes, lung, and blood (10, 15). Additionally, literatures have reported comparisons of viral loads in various organs of ASFV-infected animal models (10, 15), revealing that viral loads in the spleen, lungs, tonsils, and lymph nodes of ASFV-positive pigs were consistently high and stable. A systematic investigation was undertaken to examine 11 organs from 10 pigs, which unveiled that livers exhibited the highest viral loads, followed by spleens and inguinal lymph nodes, indicating ASFV causing immunosuppression in the host (27, 28). ASFV has been documented to possess various mechanisms to evade or dismantle the host's immune system (29). Hence, immune-related organs persist as the primary sites for the accumulation of ASFV particles. Several investigations have indicated the presence of ASFV in mandibular lymph nodes, mediastinal lymph nodes and bronchial lymph nodes of pigs infected with low-virulence ASF strains (10, 15, 30). Given the aforementioned rationales, in order to compare the differences in viral loads between superficial tissues and deep tissues of naturally ASFV-infected deceased pigs, representative deep tissues such as spleens, lungs, mediastinal lymph nodes, and bronchial lymph nodes, along with representative superficial tissues like tonsils, inguinal lymph nodes, and mandibular lymph nodes, were selected for detection and analysis. The findings of the study revealed that virus loads were significantly higher in deep tissues, specifically in the lungs and spleens. Conversely, in superficial tissues (tonsils, inguinal lymph nodes, and mandibular lymph nodes), the mandibular lymph nodes exhibited the highest virus loads, ranking second only to the lungs and spleens. However, obtaining these deep tissues requires opening up the carcasses, which often leads to

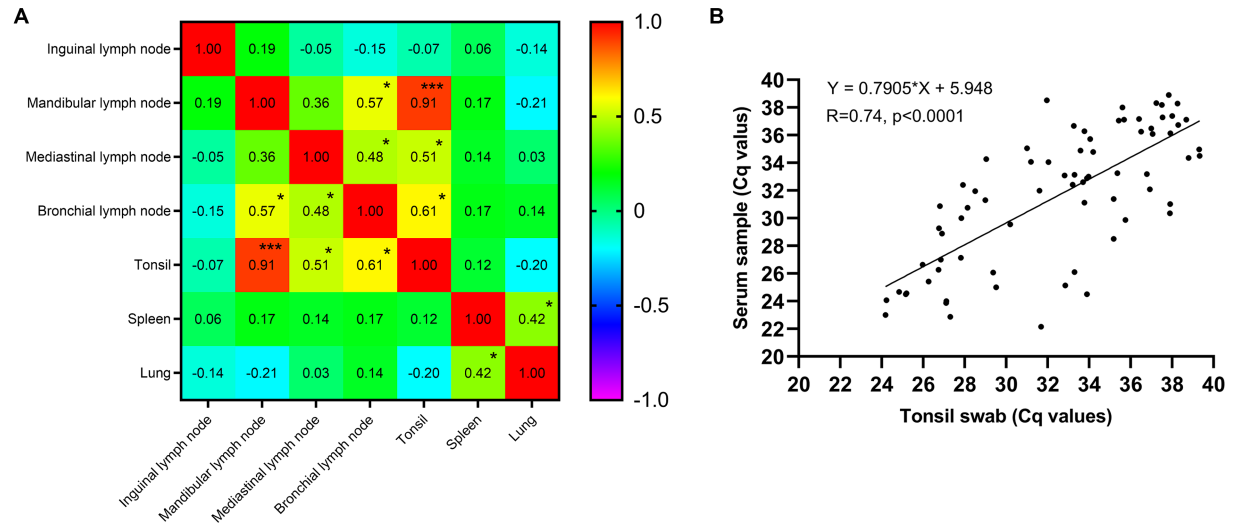

FIGURE 2
(A) The correlation coefficient matrix of ASFV genome copies between different tissues. *p value <0.001, ***p value <0.001. **(B)** The correlation coefficient between serum and tonsil swab samples.

contamination of the premises (16). Therefore, it is advisable to prioritize the collection of samples from superficial organs to ensure safe and reliable results. Pikalo et al. studied the potential of multiple superficial sample types in the passive detection of ASFV, including ocular fluids, superficial lymph nodes and ear punches, and demonstrated that superficial lymph nodes and ear punches could be potential alternatives (28). Additionally, inguinal lymph nodes have traditionally been regarded as a suitable tissue for monitoring deceased pigs, as they allow for minimally invasive sampling and minimize the risk of contamination (16, 19). This study revealed a noteworthy disparity in viral loads between mandibular and inguinal lymph nodes, with mandibular lymph nodes exhibiting significantly higher levels, which was consistent with the finds of Pikalo's (28). The mandibular lymph node is located in the mandibular space, on the inside of the lower margin of the left or right mandibular angle, in front of the submandibular gland, and is covered by the oral end of the subauricular gland (31). Furthermore, a lymph node sampler developed by our laboratory can also be used to sample mandibular lymph nodes in dead pigs (19). Consequently, this finding suggests that mandibular lymph nodes may prove to be a more suitable option for the screening of deceased pigs for ASFV in subsequent investigations.

Active monitoring primarily entails the surveillance of live pigs, typically encompassing the acquisition of serum samples, as there are a large number of virus particles in the blood in ASFV-positive pigs (11, 13, 14). Moreover, given the intricate nature of blood sampling process in pigs, the involvement of a minimum of 2–3 individuals is necessary, potentially heightening the risk of viral transmission and broadening the extent of pathogen contamination on the premise (32). Consequently, it becomes imperative to explore alternative sample types that can substitute serum samples while being easily obtainable. Several alternative blood samples have been evaluated, including oral swabs, rectal swabs, nasal swabs, feces and so on (15, 17, 19). Research has demonstrated that the genome of ASFV can be detected in the oral fluids of weaned pigs infected with highly virulent ASFV Georgia 2007/1 and moderately virulent ASFV Malta'78 strains within a period of 3–5 days after infection (33). This study has revealed that tonsils exhibit a relatively strong correlation with other tissues, and a strong correlation ($R = 0.74$) between tonsil swabs and serum samples, thus suggesting that tonsil swabs from tonsil exudate could serve as a suitable alternative sample for diagnosing ASF infection. The utilization of tonsil swabs as opposed to serum samples for collection purposes offers advantages in terms of time and cost efficiency. Our laboratory has developed a tonsil swab sampler that uses a unique short fiber villus material to absorb as much tonsil exudate as possible. When using, the long swab is inserted into the deep position of tonsils, held for a minimum of 3 s, removed, and subsequently dissolved in normal saline, which is easy to operate, and no additional assistance tools or farmers to fixed pigs are required. This tool has been widely used in farms in China. Nevertheless, it is crucial to take into account the presence of diverse ASFV strains with varying levels of virulence when monitoring tonsil swab samples. Further research is imperative to ascertain the reliability and precision of this method in monitoring pigs infected with lower virulent strains.

In summary, based on the comparison of viral loads between different tissues of deceased pigs and between different sample types of live pigs, this study identified mandibular lymph nodes as a preferable superficial tissue sample choice for screening deceased pigs, while tonsil swabs were found to be a viable alternative for diagnosing ASF infections in live pigs. However, the findings of this study are only based on samples from field conditions. More in-depth and accurate study requires more researchers and laboratories to explore in the future. Our findings provide a good laboratory research direction, and also have the potential to provide valuable insights for enhancing the efficacy of clinical prevention and control strategies against ASFV outbreaks, thereby aiding in the mitigation of associated economic losses.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because all the samples were clinical samples provided by pig farms for ASFV diagnostic detection. Then, these clinical samples were accumulated and stored in our laboratory, therefore this is a retrospective study. Written informed consent was obtained from the owners of the animals to use the clinical samples.

Author contributions

XL: Formal analysis, Writing – original draft, Writing – review & editing. ZH: Formal analysis, Writing – original draft, Writing – review & editing. XT: Data curation, Formal analysis, Software, Writing – original draft. MF: Data curation, Formal analysis, Software, Writing – original draft. QL: Data curation, Formal analysis, Software, Writing – original draft. XW: Resources, Writing – review & editing.

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Conflict of interest

XL, ZH, XT, MF, and QL were employed by Xiajin New Hope Liuhe Agriculture and Animal Husbandry Co., Ltd.

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

Publisher's note

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Identifying risk clusters for African swine fever in Korea by developing statistical models

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Introduction: African swine fever (ASF) is a disease with a high mortality rate and high transmissibility. Identifying high-risk clusters and understanding the transmission characteristics of ASF in advance are essential for preventing its spread in a short period of time. This study investigated the spatial and temporal heterogeneity of ASF in the Republic of Korea by analyzing surveillance data on wild boar carcasses.

Methods: We observed a distinct annual propagation pattern, with the occurrence of ASF-infected carcasses trending southward over time. We developed a rank-based statistical model to evaluate risk by estimating the average weekly number of carcasses per district over time, allowing us to analyze and identify risk clusters of ASF. We conducted an analysis to identify risk clusters for two distinct periods, Late 2022 and Early 2023, utilizing data from ASF-infected carcasses. To address the underestimation of risk and observation error due to incomplete surveillance data, we estimated the number of ASF-infected individuals and accounted for observation error via different surveillance intensities.

Results: As a result, in Late 2022, the risk clusters identified by observed and estimated number of ASF-infected carcasses were almost identical, particularly in the northwestern Gyeongbuk region, north Chungbuk region, and southwestern Gangwon region. In Early 2023, we observed a similar pattern with numerous risk clusters identified in the same regions as in Late 2022.

Discussion: This approach enhances our understanding of ASF spatial dynamics. Additionally, it contributes to the epidemiology and study of animal infectious diseases by highlighting areas requiring urgent and focused intervention. By providing crucial data for the targeted allocation of resources for disease management and preventive measures, our findings lay vital groundwork for improving ASF management strategies, ultimately aiding in the containment and control of this devastating disease.

KEYWORDS

African swine fever, spatial dynamics, temporal heterogeneity, generalized linear model, statistical modeling, risk clusters

1 Introduction

African swine fever (ASF) is a severe viral infection that causes hemorrhagic fever in pigs, often leading to a high fatality rate of approximately 100% (1). According to the World Organization for Animal Health (WOAH) (2), ASF is a priority disease due to its significant health and economic repercussions for swine producers and government disease control agencies. ASF infection is caused by the African swine fever virus (ASFV), which belongs to the genus *Asfivirus*, and can be transmitted through both direct and indirect pathways. Direct

transmission occurs through contact with the live bodies or carcasses of infected pigs, while indirect transmission happens via contact with contaminated objects, such as feed, water, and needles (3, 4). Some studies have utilized Geographic Information Systems (GIS) and remote sensing technologies to analyze disease spread in wildlife based on environmental factors and spatial data, confirming their potential role in monitoring and management (5–8). These studies have indicated that ASF spread can vary according to spatial characteristics.

The ASF outbreak was first documented in Kenya in 1921 (9) and became endemic in some regions of Africa. Subsequently, it spread to Europe and South America, where it was mostly eradicated. However, in 2007, the virus was introduced to Europe through Georgia (10), leading to widespread transmission. In Asia, the first case was reported in China in August 2018, followed by occurrences in other Asian countries (11–14). ASFV is typically classified based on pathogenicity into high, moderate, and low virulence. Highly virulent strains cause death within approximately 8 days, moderately virulent strains within about 20 days, and low virulence strains result in subclinical or chronic disease (4, 15). Chronically infected individuals play a crucial role in the long-term persistence of the virus, making early eradication difficult (16).

In the Republic of Korea, the first confirmed case was identified in September 2019 at a pig farm in Paju, Gyeonggi Province, and it has since continued to spread, primarily in the Gyeongbuk region (17, 18). Between October 9, 2019, and May 20, 2024, approximately 3,555 cases have been reported, involving 40 domestic pig farms and 3,515 wild boars (19, 20). Low virulence strains of ASFV have been predominantly identified in endemic regions such as Northern Europe and China. In China, the emergence of chronically infected individuals has been attributed to the production and use of illegal vaccines (21). Conversely, ASF outbreaks in the Republic of Korea have been confirmed to be caused by highly virulent strains, leading to death within 8–10 days post-infection (22, 23). Based on this information, we consider that ASF-infected carcasses reveal the overall spread patterns of ASF infection in the Republic of Korea, though they may not perfectly reflect real-time infection trends. This assumption is supported by previous studies, which also utilized carcass data to identify risk clusters for ASF outbreaks in the Republic of Korea (24).

Another characteristic of ASF spread in the Republic of Korea is that direct transmission of ASF between wild boars and domestic pigs is relatively unlikely because domestic pigs are confined in enclosed pigsties within fenced buildings, such as intensive indoor housing (25). However, ASF transmission to domestic pigs is possible via objects associated with ASF-infected wild boar or human interactions (3, 26). Accordingly, the Korean government implemented systematic and comprehensive interventions to prevent the spread of ASF, including reducing the density of wild boars, promptly disposing of carcasses, and installing fences around ASF-infection areas (27–29). To further preemptively block the spread of ASF between regions, extensive fencing was installed in six phases (stage 1, stage 2, …, stage 5–1, stage 5–2) from November 2019 to May 2022, spanning a total of 1,831 kilometers across 34 districts (3, 17, 30, 31). Several studies have confirmed the importance of active preventive measures, including the installation of fences (29, 32, 33). Notably, a study by Lim et al. (32) showed that the third phase of national fencing decreased the infection pressure on individuals in neighboring habitats by 47% compared to the same geographical habitat. This finding supports the effectiveness of fencing in limiting wild boar movement and reducing ASF transmission. However, despite these interventions and achievements, outbreaks in pig farms and wild boar populations have not been completely controlled. The continued occurrence of ASF highlights the need for a deeper understanding of the mechanisms of disease spread, emphasizing the necessity of predicting and analyzing risk clusters to improve ASF control strategies.

To understand the mechanisms of ASF spread, many studies have been conducted on factors influencing disease transmission, including the environmental and geographical factors. An analysis of the impact of environmental factors on ASF outbreaks and control revealed that the presence of roads and rivers effectively reduces the transmission rate by approximately 37% on average (34). Additionally, wild boars living at altitude above 1,000 meters are difficult to control through hunting, and the probability of transmission is higher in certain forest areas, such as the Taebaek Mountains across the Republic of Korea and the Democratic People's Republic of Korea (32). In the present study, we aimed to analyze the patterns of ASF outbreaks using the geographical coordinates (longitude and latitude) of ASF-infected carcasses, along with spatial information such as forest area, slope, and altitude. By estimating the risk clusters for emerging ASF outbreaks, this study could provide alternative approaches for developing surveillance systems.

2 Materials and methods

We conducted a statistical analysis to identify risk clusters under surveillance for ASF outbreaks by investigating the continually evolving spreading patterns of ASF based on information on ASF-infected carcasses reported in the Republic of Korea from October 2019 to April 2023. Using Standard Deviation Ellipse (SDE) analysis and rank-based method, we aimed to identify regions at risk of ASF outbreaks. Figure 1 illustrates the statistical analysis approach used to identify risk clusters. We assessed the direction and variance of the spread using the SDE to comprehend the spatial transmission patterns at each time point. Furthermore, we constructed a generalized linear model (GLM) to compute the number of wild boar carcasses in the region for each period using the designated probability distribution. We developed a statistical model using a rank-based method to evaluate ASF risk. This model estimates the average weekly number of carcasses by district over time, allowing us to analyze and identify risk clusters. To mitigate the underestimation of risk and account for observation errors caused by undetected ASF-infected individuals, we estimated the number of ASF-infected animals and incorporated observation error by considering different surveillance intensities.

2.1 Data analysis

2.1.1 Data description

The spatial distribution of this surveillance data is illustrated in Figure 2A. The surveillance data on wild boar carcasses infected with ASFV from October 2019 to April 2023 in the Republic of Korea was

Abbreviations: ADF, Augmented Dickey– Fuller; ASFV, African swine fever virus; ASF, African swine fever; FMD, Foot and mouth disease; GLM, Generalized linear model; HT season, High transmission season; LT season, Low transmission season; MAFRA, Ministry of agriculture, food, and rural affairs; NB, Negative binomial; SDE, Standard deviation ellipse; WOAH, World Organization for Animal Health; ZIP, Zero-Inflated Poisson.

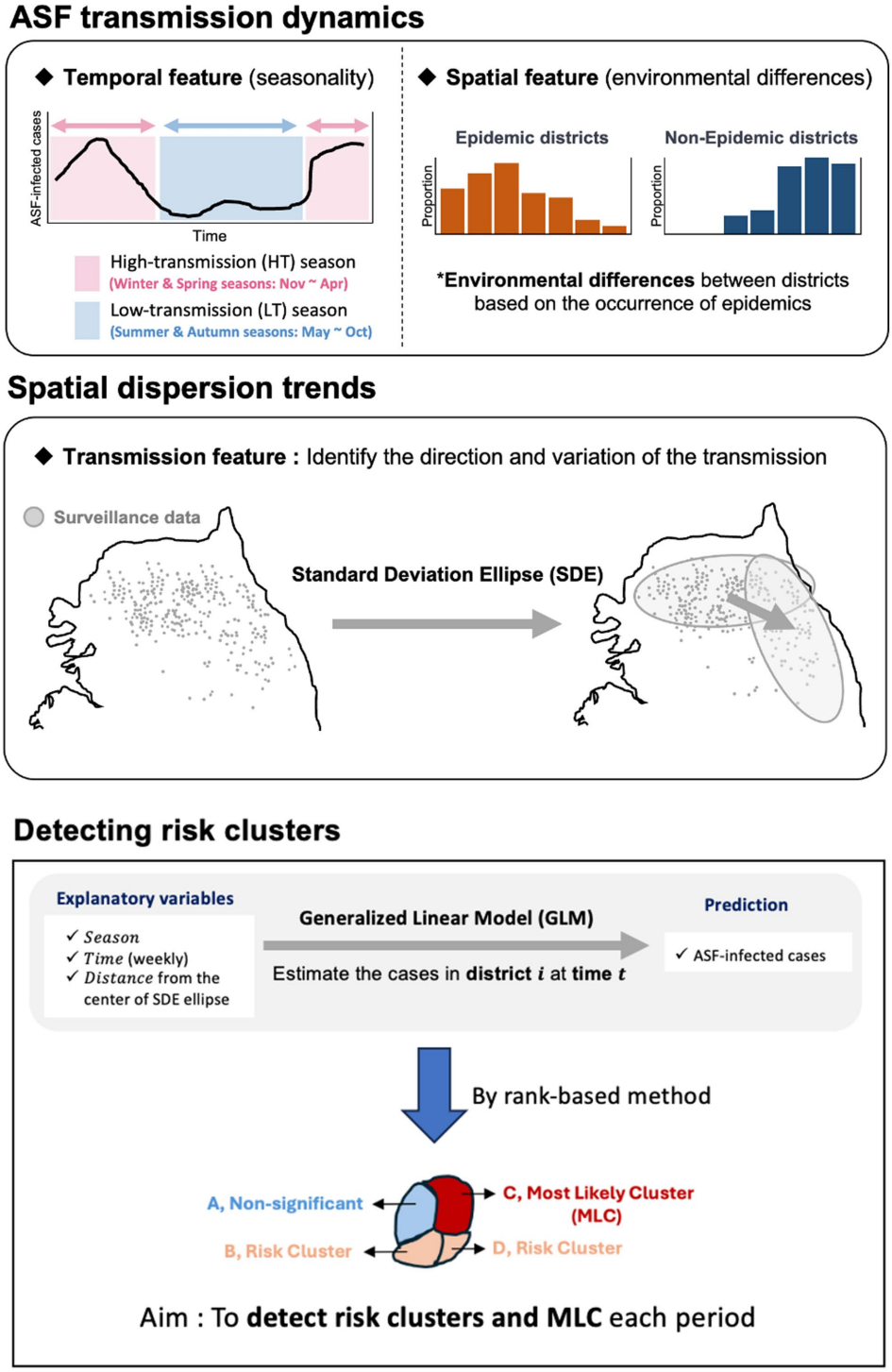

FIGURE 1
An outline for identifying risk clusters and most likely clusters (MLC) using rank-based score.

analyzed. This dataset, compiled from data provided by the Ministry of Agriculture, Food, and Rural Affairs (MAFRA) (20) and supplemented with information from the ASF real-time status board derived from the *Google Map* service (35), includes diagnosis dates, observation dates, observation methods (including carcass removal and hunting), geographical coordinates (longitude and latitude), and the locations of the collected samples. Observation dates denote when carcasses were discovered, while diagnosis dates indicate the confirmation of an ASFV-positive diagnosis.

The administrative boundary data in the Republic of Korea comprises approximately 250 districts and 17 regions. In this study, a "district" is defined as a city, county, and borough, while "area" refers to several cities. To analyze the trends in the spread of ASF, we collected information on the installation of extensive fencing and

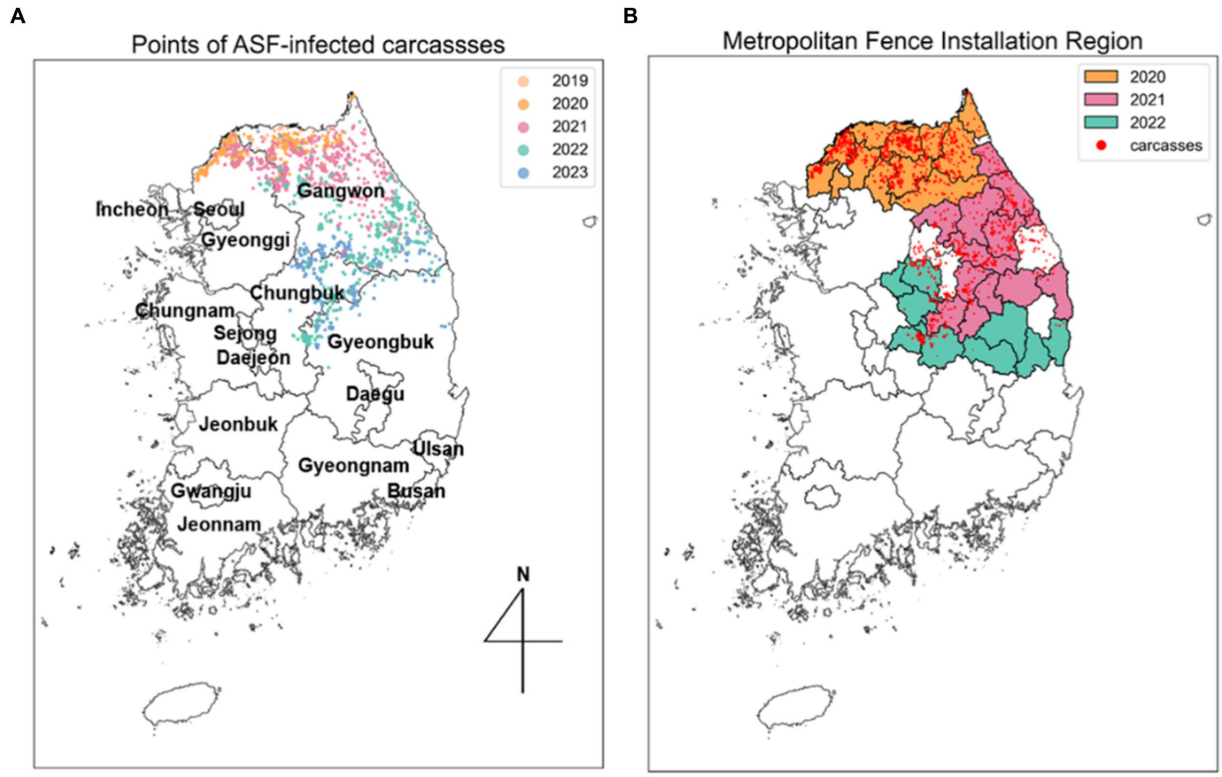

FIGURE 2
Spread trend of ASF outbreaks from SDE. **(A)** Five different colors indicate the ellipses obtained from SDE by year. Points, representing the observed locations of carcasses for each year, are colored to match the corresponding year's ellipse. **(B)** Each colored area outlines the regions where metropolitan fences were installed each year, corresponding to the same color scheme used for the ellipses.

environmental data, including forest area (m^2), elevation (altitude), and slope data by district, all classified by administrative boundaries. Extensive fencing data, sourced from the Ministry of Environment (17), indicates that 1,831 kilometers of fencing were installed across 34 districts. To interpret the effectiveness of these fencing control measures, we categorized the areas with additional fencing installed annually from 2020 to 2022 and organized this along with the ASF-infected carcass data, as shown in Figure 2B. The ASF-infected carcasses data and environmental data for Gyeonggi and Gangwon regions are summarized in Supplementary Table S1.

2.1.2 Estimation of ASF-infected counts from ASF-infected carcass data

ASF-infected carcass information serves as useful indicators of infection spread and is therefore used as primary data. Many previous studies have utilized ASF-infected carcass data to understand the dynamics of ASF (18, 36, 37). However, due to potential underestimation of the outbreak scale caused by delays in carcass detection and the period between becoming infectious and death, ASF-infected carcass data may be insufficient to accurately reflect the actual spread of infection. Thus, we estimated the number of infected individuals (estI) over time and used this estimation as comparative data to the observed number of ASF-infected carcasses (obsC) to more accurately represent true infections that could impact actual transmission.

ASFV found in the Republic of Korea from 2019 to 2023 is typically highly pathogenic, causing death in infected pigs within a week (18, 23). Infection experiments conducted in the Republic of Korea revealed that the estimated time to virus detection was 3.7–4.8 days, the incubation period was between 3.4 and 5.2 days, and the time to death was 8.9 to 9.1 days (38). Based on these findings, we assume that the time from infection to carcass discovery follows a uniform distribution with a mean of 9 days and a range between 0 and 18 days. Throughout the entire time, we estimated estI by back-calculating from the obsC. This process involved estimating the time interval between the observation dates of obsC and the estimated infection dates, which were randomly generated from a Uniform (0, 18) distribution, allowing us to estimate estI over time. We compared the risk clusters identified using obsC and estI to verify differences in risk assessment. This comparative approach is expected to preemptively identify risk clusters and enable more proactive predictions compared to relying solely on carcass-based observations.

2.2 Spatial and temporal dynamics of ASF transmission

Observational data have revealed that ASFV infections are more prevalent during the winter and spring seasons, which coincide with the wild boar breeding season (39), contrasting with lower frequencies of infections observed in summer and autumn (32). Accordingly, we have stratified the year into two distinct seasons for analysis: the "high transmission season (HT season)," spanning from November to April, and the "low transmission season (LT season)." This division allows for a systematic investigation of the temporal heterogeneity and transmission dynamics of ASF. The distinction is supported by the observation that about 80% of ASF cases in the Republic of Korea

occurred during the HT season from 2020 to 2022. Additionally, we categorized the 250 districts into two groups: epidemic districts, where ASF cases have been reported, and non-epidemic districts, where no cases have been reported. This analysis particularly focused on the Gyeonggi and Gangwon regions, which account for over 80% of the carcass count.

We conducted statistical hypothesis tests to analyze temporal variations in carcass counts between HT and LT seasons and distributional differences of environmental factors such as forest area, elevation, slope between epidemic and non-epidemic districts.

First, we employed the augmented Dickey–Fuller (ADF) test to examine the stationarity of cumulative ASF-infected carcasses during the HT and LT seasons. The ADF test serves as a statistical tool to determine the stationarity of time-series data based on the null hypothesis that stationary data do not maintain constant statistical properties over time (40). This approach is particularly relevant for identifying temporal variations in ASF transmission rates, offering a statistical basis to assess the effects of seasonality on the spread of ASF.

Second, we applied non-parametric tests including the Ansari–Bradley and Mann–Whitney U tests to investigate distributional differences in forest area, slope, and elevation (altitude) between the epidemic and non-epidemic districts. Higher *p*-values from the Ansari–Bradley test indicate variability in dispersion patterns, while lower *p*-values from the Mann–Whitney U test highlight significant differences in central tendencies. These tests were selected because of their efficacy in managing the nonnormal distribution of data, thereby enabling a robust comparison of the variances and median values between the two district groups (41, 42).

Through this methodological approach, we aimed to enrich the comprehension of the spatial and temporal analysis conducted in our study, subsequently providing insights into the unique transmission characteristics of ASF in the Republic of Korea, described within specific seasonal and geographical contexts.

2.3 Statistical modeling for estimating the number of carcasses

2.3.1 Standard deviation ellipse

The SDE is a spatial statistical tool widely used to describe the directional trend and dispersion of geographical features in spatial distribution, utilizing the longitudinal and latitudinal locations of ASF-infected carcasses (43, 44). Historically, SDE has been used to analyze spatial dispersion and directional bias in Poland (45) and to investigate the directional trend and spread of Foot and Mouth Disease (FMD) in China (46). Building on these applications, we employed the SDE method using observed carcass data to identify annual and monthly changes in occurrence regions of ASF in the Republic of Korea. This method facilitates the creation of ellipses that capture spatial characteristics such as orientation, spatial dispersion, and directional trends of ASF outbreaks.

The outcomes of the SDE method include the lengths of longitudinal and latitudinal axes, their ratio, angle, and the center point. The lengths of the longitudinal and latitudinal axes quantify the dispersion in the east–west (horizontal) and north–south (vertical) directions, respectively, based on the variance of the obsC data used for measurement. Moreover, the angle of the ellipse, determined by the longer axis, indicates the principal direction of data spread, starting from north and moving clockwise. This reveals the direction in which the spatial dispersion pattern of the data tends. The SDE ratio, a longitude-to-latitude measure, indicates the ellipse's deviation from a circular shape based on the lengths of the longitudinal and latitudinal axes (44). A ratio between zero and one indicates a vertical dispersion tendency, whereas a ratio greater than 1 implies a horizontal dispersion tendency. As the axis lengths approach equality, nearing a ratio of one, the ellipse tends to resemble a circle, suggesting limited propagation in a specific direction.

The dimensions of these ellipses, represented by their long and short axes, are defined by the variances in longitude and latitude of the observed carcasses. The size of the ellipse, adjustable based on the carcass count, is governed by the variance in both dimensions (47, 48), and the angle of the ellipse is derived from the covariance between longitude and latitude. Using sigma (σ) to denote the standard deviation for both longitude and latitude, a single sigma (1σ) along each axis typically encompasses approximately 66.7% of all carcasses within the ellipse. Two sigma (2σ) captures about 95.5%, and three sigma (3σ) includes approximately 99.7% of the total carcasses (48, 49).

2.3.2 Effect of the surveillance intensity on ASF-infected carcasses

Surveillance data on ASF-infected carcasses is collected through a government-implemented carcass collection policy, and this data is crucial for analyzing the response to the ASF outbreaks. Typically, surveillance and control policies, such as installing fences to prevent spread, are initiated in districts where ASF-infected carcasses are found (17). Considering the limited resources available for response policies, the intensity of response may vary according to the outbreak severity in different areas. It is expected that surveillance will be intensified in districts with a higher number of ASF-infected carcasses discovered. Conversely, districts with lower surveillance intensity are likely to have a higher number of undiscovered infected carcasses. This difference between the actual and reported number of infected individuals, defined as the observation error, can vary with the surveillance intensity.

To understand the impact of surveillance intensity, we conducted a scenario analysis focused on observation errors. We employed a spatial dispersion analysis of ASF spread using SDE analysis of ASF-infected carcass data collected during the observation period to delineate surveillance areas. Surveillance intensities were adjusted across different zones of the ellipse based on the standard deviation (sigma) setting. These zones included 66.7% of the data within the 1-sigma ellipse, 95.5% within the 2-sigma ellipse, and 99.7% within the 3-sigma ellipse, with surveillance intensity decreasing progressively from the 1-sigma to the 3-sigma ellipse.

We calculated the adjusted number of ASF-infected carcasses ($adjC_{i,t}$) in district i at time t from the $obsC_{i,t}$ through different observation error rates ($\varepsilon\%$), determined by the intensities of surveillance, as follows:

$$adjC_{i,t} = obsC_{i,t}\left(1+\varepsilon/100\right).$$

The $obsC_{i,t}$ are set according to designated observation error rates at each stage of surveillance intensity as follows:

- **(Strong intensity)** In 1-sigma districts, the surveillance is conducted at maximum intensity with an observation error of 0%, implying that $adjC_{i,t}$ equals the observed number:

$$adjC_{i,t} = obsC_{i,t}$$

- **(Intermediate intensity)** In 2-sigma districts, the observation error is defined by a specific factor, adjusting the observed number to estimate the total number as:

$$adjC_{i,t} = obsC_{i,t}\left(1+\varepsilon/100\right)$$

- **(Low intensity)** In 3-sigma districts, the observation error is twice that of 2-sigma districts:

$$adjC_{i,t} = obsC_{i,t}\left(1+2\,\varepsilon/100\right)$$

- **(Not implemented)** Surveillance is not implemented outside the 3-sigma ellipse, where ASF occurrences are negligible, covering only 0.3% of the observation data.

2.4 Analysis of the generalized linear model

We employed a GLM to estimate the weekly number of ASF-infected carcasses across 250 districts in the Republic of Korea over time. The GLM extends the linear regression model to accommodate response variables following various probability distributions, such as Poisson, Negative binomial, and Zero-inflated distributions, by connecting them with the response variable through a link function, $f(\cdot)$. In our model, we assumed the number of carcasses, Y_{it} in district i at time t, adheres to these designated distributions. The GLM to estimate the number of ASF-infected carcasses ($estC_{i,t}$) in district i at time t, is formulated as:

$$f\left(Y_{it}\right) = \alpha + \beta_1\, Distance_{i,t} + \beta_2 Distance_{i,t}{}^2 + \beta_3\, Season_t + \beta_4\, Time_t$$

Where $\beta_1, \beta_2, \beta_3$ *and* β_4 represent the coefficients for each variable, and α is the intercept. The outcomes of the SDE analysis revealed spatial features that were previously unconsidered in the regression equation. The Distance variable represents an exponential decay of the Euclidean distance, thereby assigning higher transmission risk to closer districts. By incorporating these insights, we defined the *Distance* variable for district i at time t as $Distance_{i,t} = \exp\left(-d_{ij}\left(t\right)\right)$. Here, $d_{ij}\left(t\right)$ represents the Euclidean distance between district i and the center point j of the SDE ellipse at time t. j is defined as the center point of the SDE ellipse using carcasses data from 2022 if $t \in 2022$ or using carcasses data from 2023 if $t \in 2023$.To capture any non-linear relationships between geographical distance and obsC, we also introduced the $Distance^2$ variable.

The *Season* variable serves as an indicator variable to distinguish between HT and LT seasons, reflecting seasonal variations in ASF occurrence. Additionally, the *Time* variable is introduced to track the progression of weeks during the study period, starting from one and sequentially increasing, which helps incorporate temporal dynamics into our analysis (i.e., $Time = 1, \ldots, 16$ for Late 2022 and $Time = 1, \ldots, 17$ for Early 2023). By integrating these variables, our model captures the heterogeneity of ASF dynamics across different geographical regions and over time. This comprehensive approach enhances our ability to predict ASF spread more accurately in the Republic of Korea.

This approach, supported by previous research (50), assumes that carcass counts follow designated distributions, with Poisson regression widely used in epidemiology for analyzing count data (51, 52). However, the Poisson distribution's assumption of equal mean and variance may not always align with real-world data, prompting us to include models based on Negative Binomial (NB) and Zero-Inflated Poisson (ZIP) distributions. This expansion, inspired by prior research including studies on pig infection counts in Sardinia (51), aims to overcome the limitations of the Poisson model. Our analysis suggests that outcomes can vary depending on the model employed to estimate the ASF-infected carcass counts. This variability allows for a range of estimation outcomes influenced by the differential impact of diverse distributions on the coefficients of the independent variables. To facilitate this analysis, we generated the estimates from each distribution according to the estimated coefficients, thereby enabling a comparison. This approach offers a comprehensive analysis of ASF outbreak data using various distributions and provides a deeper understanding of the dynamics of ASF outbreaks.

This analysis was implemented using the R programming language. To fit the GLM models, we used the "glm" function from the "stats" package for Poisson distributions, the "glm.nb" function from the "MASS" package for NB distributions, and the "hurdle" function from the "pscl" package for a ZIP distributions.

2.5 Identifying risk clusters of ASF outbreaks

We conducted our analysis during the HT period to identify risk clusters for two periods: September to December 2022 (Late 2022) and January to April 2023 (Early 2023). When ASF occurrence in a specific area surpasses the GLM-based estimate, it suggests either randomness or a higher than expected level of occurrence, signaling the need for heightened attention to that area. However, since surveillance data is collected based on the discovery of carcasses of infected individuals rather than the real-time number of infected cases, identifying risk clusters by simply comparing the surveillance data with the estimated counts is less effective. Instead of focusing solely on carcass counts, we compared the rankings of areas with a high risk of ASF occurrence between observed data and estimates from GLM. Specifically, we ranked areas based on the obsC over a week to identify regions with high outbreak risk, while simultaneously ranking areas based on predicted carcass counts to determine expected risk districts. Furthermore, we used the Mann–Whitney U test, also known as the Wilcoxon rank-sum test, which is a non-parametric method for comparing the medians between two groups, to evaluate whether the actual rank is significantly higher than the estimates. Subsequently, we defined a risk cluster as one where the rank of the actual risk cluster is significantly higher than the rank of the

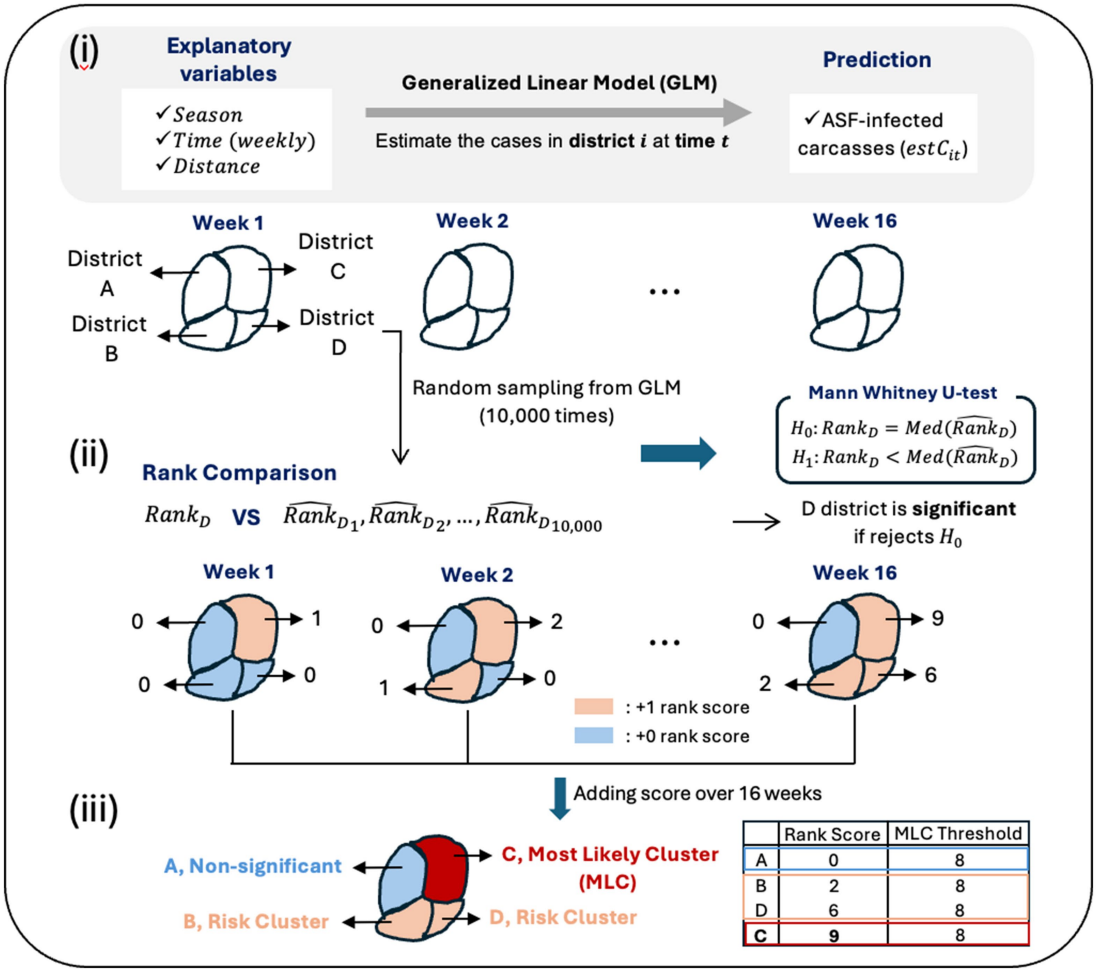

FIGURE 3
Outline for detecting the risk clusters of ASF outbreaks.

expected risk cluster. We elucidated the following procedures to identify risk clusters for ASF outbreaks, described in Figure 3:

(i) Estimation of the ASF case counts using GLM: Given the observed number of ASF-infected carcasses ($obsC_{i,t}$), we estimated the number of ASF-infected carcasses ($estC_{i,t}$) in district i and week t, where i = 1, …, 250 and t = 1, …, 16 for Late 2022 and t = 1, …, 17 for Early 2023. Here, we assumed that ASF-infected carcass counts follow a designated distribution. To ensure the robustness of our estimates, we generated 10,000 random samples $s_{i,t}^{(1)}, \cdots, s_{i,t}^{(10000)}$ from the designated distribution with parameters $\lambda_{i,t}$ for each district i and time t.

(ii) Rank score by Rank-based method and Mann Whitney U-test: We assigned ranks to each of the 250 districts based on the $obsC_{i,t}$ for each week t, denoted by $R_{1,t}, \cdots, R_{250,t}$. Similarly, for all samples, we assigned ranks across the 250 districts through comparison. For example, in the case of the nth sample across the 250 districts, $s_{1,t}^{(n)}, \cdots, s_{250,t}^{(n)}$, assigned ranks included $r_{1,t}^{(n)}$, …, $r_{250,t}^{(n)}$. Subsequently, we calculated the median rank for each district based on the ranks assigned to all samples, with the median rank for district i and week t denoted as $\hat{R}_{i,t}$. In other words, the ranks generated for each sample in district comparison for district i and week t, resulting in a total of 10,000 ranks $r_{i,t}^{(1)}, \ldots, r_{i,t}^{(10000)}$, are used to find the median value as $\hat{R}_{i,t}$. Based on the measured ranks for a specific district i and time t, $R_{i,t}$ and $\hat{R}_{i,t}$, we constructed the null hypothesis as $H_0 : R_{i,t} = \hat{R}_{i,t}$ and the alternative hypothesis as $H_1 : R_{i,t} < \hat{R}_{i,t}$. Then, we could determine the significance by examining the p-values. If the p-value is less than 0.05, it is concluded that the actual carcass rank is significantly higher than the estimated carcass rank.

(iii) Risk clusters and the most likely clusters: The rank-based score (rank score) is defined such that if the Mann–Whitney U-test result is significant at the 0.05 level of significance, we assign a score of 1 to district i at time t. For Late 2022, there are 16 time points, and for Early 2023, there are 17 time points. Therefore, the maximum rank score for each district i is 16 for Late 2022 and 17 for Early 2023. Here, we defined the risk clusters as those where the rank score is greater than 1. In other words, a district with a rank score greater than 1 indicates that at least one time point was significant, indicating that the actual risk rank was

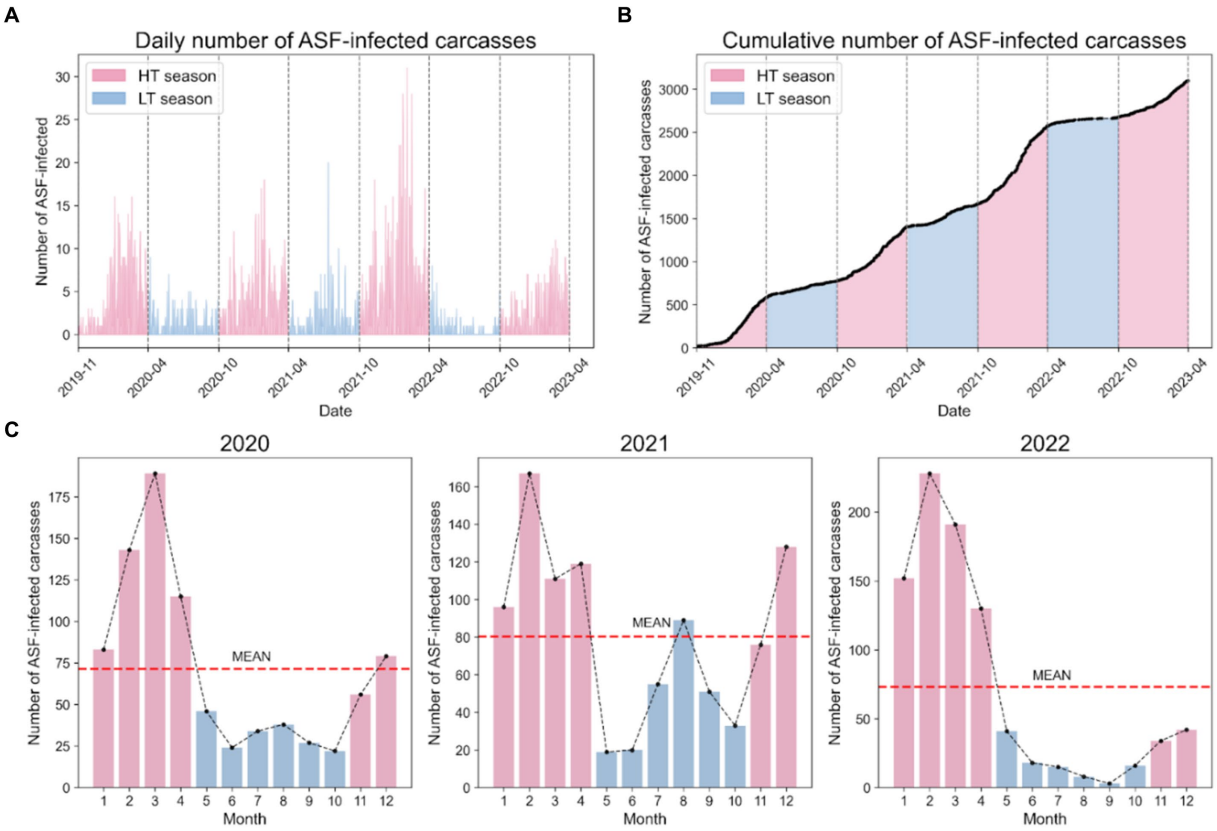

FIGURE 4
Temporal dynamics of ASF outbreaks in the Republic of Korea. **(A)** The daily cases of ASF-infected carcasses observed from 2019 to 2023. **(B)** The cumulative cases of ASF-infected carcasses observed from 2019 to 2023. **(C)** The observed instances of ASF-infected carcasses in the Republic of Korea are presented annually from 2020 to 2022. Bars in blue represent the LT season, while red bars represent the HT season. The red dashed line represents the average number of ASF-infected carcasses for that year.

higher than expected from GLM at least once. Additionally, areas where more than half of the time points within the given period are significant, resulting in a rank score is 8 or higher, are designated as the most likely clusters (MLC).

3 Result

3.1 Statistical analysis of the spatial and temporal dynamics of ASF transmission

Figure 4 shows the *obsC* data for the spatiotemporal distribution of ASF occurrences across the Republic of Korea from November 2019 to April 2023. The figure highlights the temporal dynamics of ASF outbreaks throughout the observation period, distinguishing between the HT and LT seasons to verify ASF seasonality. Figures 4A,B provide a detailed account of the daily and cumulative number of cases, reporting 2,698 ASF cases. During the HT seasons, there were 2,139 discovered carcasses, approximately 3.82 times higher than the 559 carcasses identified during the LT seasons, as shown in Figure 4A. This discrepancy is further highlighted by a more pronounced increase in cumulative cases during the HT season, as depicted in Figure 4B. Moreover, Figure 4C illustrates that the monthly instances during the LT season were significantly lower compared to the monthly averages of 71 in 2020, 80 in 2021, and 73 in 2022, emphasizing a more rapid spread of ASF throughout the Republic of Korea during the HT seasons.

The ADF test was used to evaluate the stationarity between the HT season and the LT season based on the cumulative number of ASF-infected carcasses. The ADF test yielded a p-value of 0.9025, exceeding the threshold of 0.05, thereby not rejecting the null hypothesis of the test, which posits that the time-series data are nonstationary. This indicates that the mean or variance of the data on the ASF spread may vary over time. Consequently, through our comprehensive analysis of seasonality, we elucidated the temporal heterogeneity in the presence of ASF in the Republic of Korea.

The environmental data used to analyze spatial characteristics and potential risk factors by administrative districts include forest area data collected from the Korea Forest Service shown in Figure 5A, and elevation and slope data extracted using QGIS (version 3.26.2) shown in Figures 5B,C. Between 2019 and 2023, 81.01% of all ASF-infected carcasses were found in the Gangwon and Gyeonggi regions in the Republic of Korea. Figures 5D–F provide a monthly comparison of the forest area, elevation, and slope data between the epidemic and non-epidemic districts within the Gyeonggi and Gangwon regions. The data shows that epidemic districts exhibited significantly higher metrics in terms of forest area, elevation, and slope compared to non-epidemic districts.

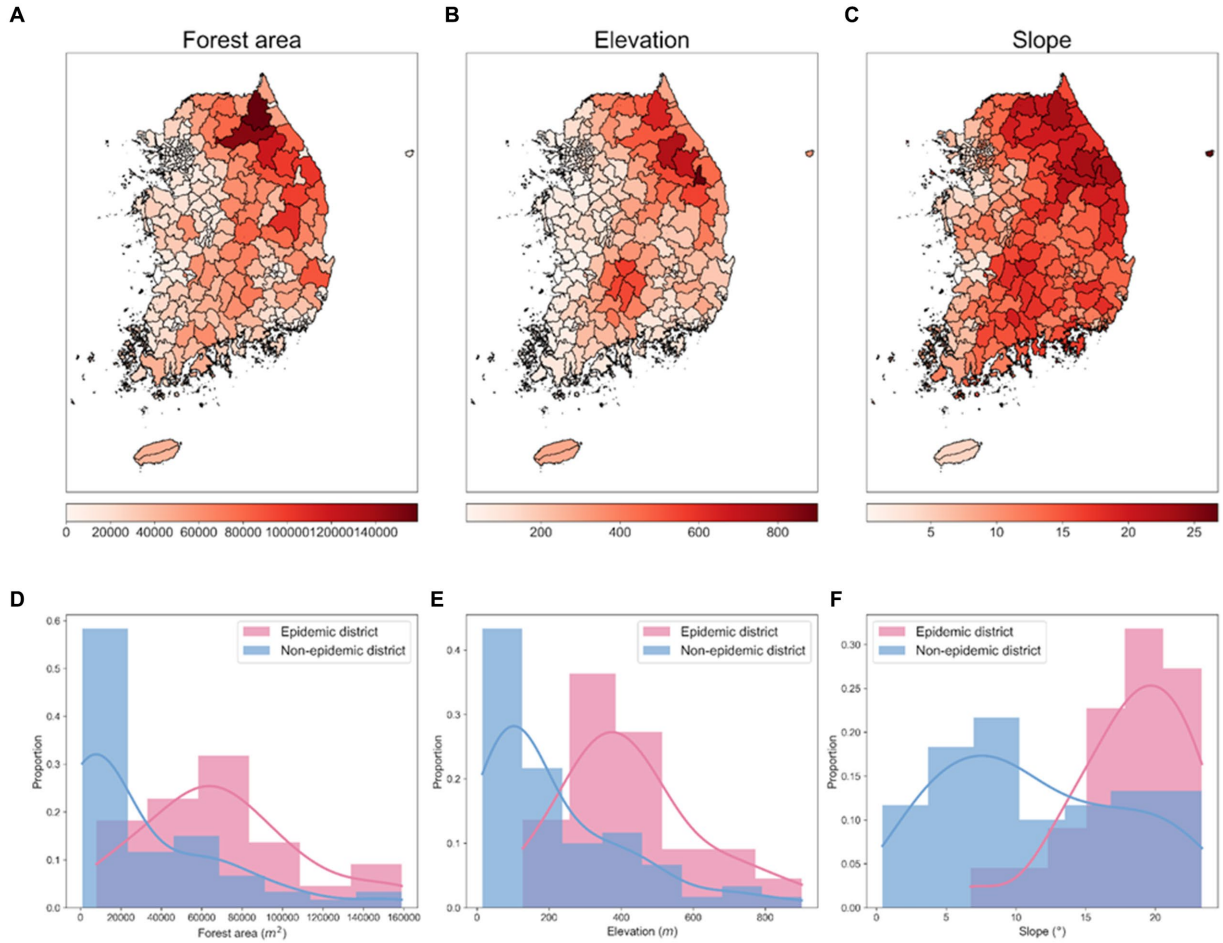

FIGURE 5
Spatial distributions of environmental factors and their proportional distributions in epidemic and non-epidemic ASF districts. **(A)** Spatial distribution of forest area (in m^2). **(B)** Spatial distribution of elevation (in m). **(C)** Spatial distribution of slope (in °). Each visualization represents an area approximately 667 km vertically and 542.46 km horizontally. **(D–F)** Proportional distribution of forest area, elevation, slope in epidemic and non-epidemic districts, respectively. The shaded area represents the histogram of the data, and the solid line represents the density curve.

Correlation analysis further supported the geographical influence on the spread of ASF, as depicted in Supplementary Figure S1. There was a notable difference in the correlation coefficients between environmental factors and carcass counts when comparing all districts to the specific regions of Gyeonggi and Gangwon. For all districts, the correlation coefficients between carcass counts and elevation, slope, and forest area were 0.50, 0.46, and 0.48, respectively (Supplementary Figure S1A). However, these correlations were significantly higher in the Gyeonggi and Gangwon regions, with coefficients of 0.75 for elevation, 0.75 for slope, and 0.78 for forest area (Supplementary Figure S1B).

To quantify the distributional differences between epidemic and non-epidemic districts, we conducted the Ansari–Bradley test and the Mann–Whitney U test of non-parametric statistical tests. The higher *p*-values in the Ansari–Bradley test suggest variability in dispersion patterns, whereas the lower *p*-values from the Mann–Whitney U test point to concrete disparities in central tendencies. The results from the Ansari–Bradley test produced *p*-values of 0.371 for forest area, 0.095 for elevation, and 0.221 for slope, surpassing the threshold of 0.05, suggesting variance discrepancies across all environmental factors between the epidemic and non-epidemic districts. Conversely, the Mann–Whitney U test yielded *p*-values of 0.002 for forest area, 0.004 for elevation, and 0.005 for slope, all below 0.05, denoting significant distributional differences in the median values for the factors assessed. These findings highlight the distinct distributional differences of environmental factors between the epidemic and non-epidemic districts. Specifically, the forest area was highlighted as a critical factor in challenging the null hypothesis. This comprehensive analysis of environmental factors elucidated the spatial heterogeneity of ASF in the Republic of Korea and affirmed the interplay between geographical features and ASF distribution patterns.

3.2 Analyzing the southward trend of ASF using standard deviation ellipse

Figure 6A illustrates the annual directional trend and dispersion characteristics of ASF spread from 2019 to 2023, using the SDE method. Table 1 reveals that during this period, longitude-axis changes were minimal, ranging from 2.7709 to 1.3462, while latitude-axis exhibited a significant increase, from 0.5672 to 2.4875. This indicates a notable evolution from horizontal to vertical

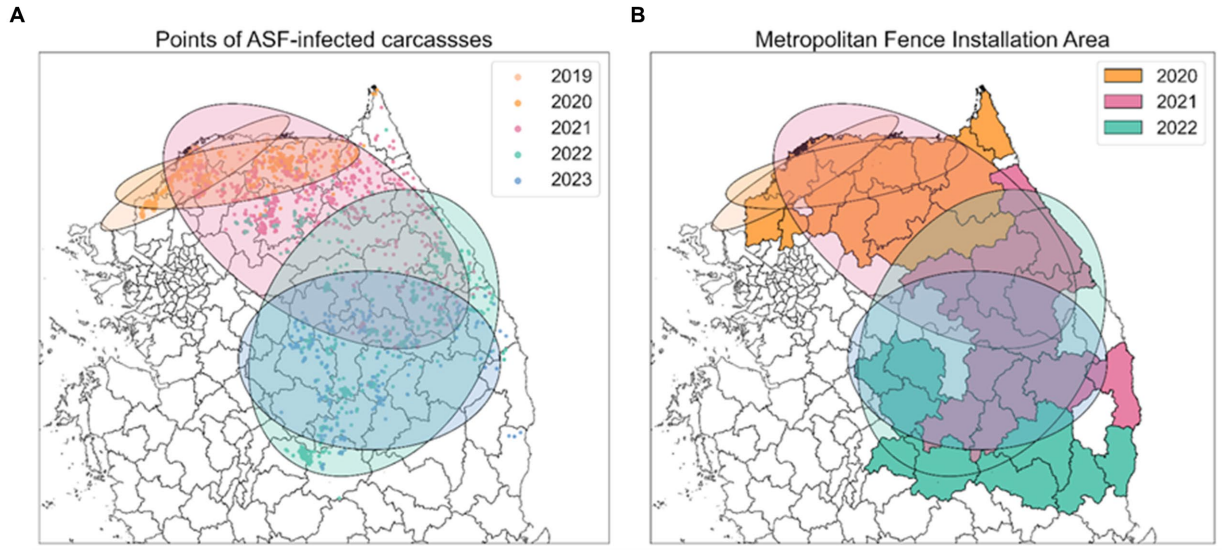

FIGURE 6
Spatial distribution of ASF-infected carcasses in the Republic of Korea. **(A)** Points indicate yearly observed ASF-infected carcasses from 2019 to 2023. **(B)** Areas where fences were newly expanded from 2020 to 2022. Red dots indicate observed ASF-infected carcasses.

TABLE 1 Geographical information of Standard Deviation Ellipse by year.

Year	Longitude-axis	Latitude-axis	Ratio*	Angle	Center (latitude, longitude)
2019	2.77090	0.56721	4.8854	65.4516	(127.096, 38.111)
2020	2.51847	1.28738	1.9562	82.0699	(127.392, 38.111)
2021	1.34621	2.48751	0.5412	109.5758	(127.945, 37.809)
2022	1.71909	2.24605	0.7654	193.6745	(128.349, 37.200)
2023	1.98169	2.01814	0.9819	90.5783	(128.318, 37.043)

*Ratio indicates the longitude-to-latitude ratio.

dispersion patterns, highlighting southward shifts in the spread of ASF. Initially, in 2019, the SDE ellipse demonstrated a significant horizontal dispersion, particularly towards the northeast, as indicated by a ratio of 4.8854 and an angle of 65.4516. By 2020, this pattern had shifted more directly eastward. In subsequent years, vertical dispersion became more prominent, with ratios of 0.5412 in 2021 and 0.7654 in 2022. Specifically, in 2021, the dispersion tilted southeast (angle 109.5758), whereas in 2022, it turned towards the southwest (angle 193.6754), indicating a continuous southward movement of ASF. By 2023, the SDE ratio approached 0.9819, suggesting a nearly equal dispersion in all directions and highlighting the absence of any predominant dispersion tendency in any specific direction.

Figure 6B displays the expansion of fencing areas from 2020 to 2022. As the discovery of ASF-infected carcasses gradually moved southward, the large-scale fencing was extended towards the south to block further spread. During the observation period, the positioning of the 2023 SDE ellipse directly above the fencing installed in 2022, indicates that these barriers effectively influenced the containment of ASF's southward expansion. This representation highlights the strategic placement of extensive fences in response to the shifting dispersion patterns of ASF, underscoring their role in mitigating the geographical spread of ASF.

We extended the SDE analysis by incorporating its results into a GLM analysis, assuming a designated distribution to estimate the number of ASF-infected carcasses across various districts during the observation periods. As detailed in Table 2, the analysis, based on a Poisson distribution GLM, used the dataset from 2019 to 2022 as the training set, enabling a comprehensive evaluation of the variability in ASF spread across districts. The analysis revealed that the coefficient for the *Time* variable was −0.005 (i.e., $e^{-0.005} \approx 0.9950$), suggesting that the impact of *Time* on carcass count estimates was minimal. However, the *Season* variable showed a coefficient of approximately 1.9 ($e^{0.648} \oplus 1.9117$), indicating that during the HT season, the impact on carcass estimates was approximately double that of the LT season. The *Distance* variable, calculated as the negative exponent of the Euclidean distance derived from geographical coordinates, had a coefficient of 25.18. This signifies a substantial increase in carcass count as the distance decreased, confirming that closer proximity correlates with higher ASF-infected carcass detection.

3.3 Identifying the risk clusters of ASF outbreaks by using rank-based method

Due to obsC not reflecting the delay between infection and carcass discovery, it does not accurately represent real-time infection status. To address this, we estimated the estI based on obsC. Assuming a period of 9 days from infection to death, we set the infection date prior

TABLE 2 The result of Poisson regression.

Variables	Estimate	Standard Error	Z value	*p*-value
Intercept	−202.8000	0.2328	−87.10	<2e-16**
Time	−0.0050	0.0004	−11.56	<2e-16**
Distance	25.1800	0.7813	32.22	<2e-16**
Distance^2	−16.7200	0.6452	−25.92	<2e-16**
Season	0.6480	0.0413	15.68	<2e-16**

**Signifies that the variable is statistically significant within the Poisson regression model, as indicated by the coefficient of variable yielding p-value below 0.05.

to the observation date of obsC using a uniform distribution with a mean of 9 days. Supplementary Figure S2 illustrates the comparison between estI and obsC over time from October 2019 to April 2023. Subsequently, we applied a rank-based method incorporating the Poisson distribution to identify ASF risk clusters based on both estI and obsC, as depicted in Figure 7. This comparative approach facilitates more efficient identification of risk clusters compared to methods that rely solely on obsC.

We conducted an analysis to identify risk clusters for two distinct periods, Late 2022 and Early 2023, utilizing data from ASF-infected carcasses (Figures 7A,B) and estimates of infected individuals (Figures 7C,D). Risk clusters are marked in red on the map, with districts of higher rank scores shown in deeper shades, indicating a greater risk level. Districts achieving a rank score of 8 or above are classified as MLC and bordered with light green color. Additionally, we compared districts with high carcass counts to those based on rank scores, as detailed in Table 3 and Supplementary Figure S3.

In Late 2022, the risk clusters identified by obsC and estI were almost identical, particularly in the northwestern Gyeongbuk region, north Chungbuk region, and southwestern Gangwon region (Figures 7A,C). No districts met the MLC criteria using obsC (Figure 7A); however, based on estI, the Mungyeong district (A10) in the northwestern Gyeongbuk region was confirmed as an MLC (Figure 7C). In Early 2023, we observed a similar pattern with numerous risk clusters identified in the same regions as in Late 2022 (Figures 7B,D). Additional risk clusters were discovered in the northeastern Gyeongbuk region, northwestern Chungbuk region, and southeastern Gangwon region, leading to an expansion of identified risk clusters and MLCs. The eastward movement of wild boars in 2022 was also noted. Using obsC, only the Wonju district (A2) in the southwestern Gangwon region was identified as an MLC. In contrast, analysis using estI identified a total of five districts, including the northeastern Chungbuk region and western Gyeongbuk region, as meeting the MLC criteria. Although the identified risk clusters were generally similar between the two data sources, rank scores estimated using estI were observed to be 2–3 points higher on average than those based on obsC. This suggests that relying solely on carcass data might underestimate the actual risk level. Furthermore, although districts with high carcass counts did not always align with the risk clusters, four out of the top five districts with high carcass counts were included as top five risk clusters based on rank score in both Late 2022 and Early 2023 (Table 3). Additionally, the district with the highest incidence was classified as an MLC. These results underscore that the identified risk clusters, which require significant monitoring, consistently included districts with high incidence rates throughout the observation period.

To enhance the robustness of our analysis, we explored outcomes based on obsC across various distributions: Poisson, NB, and ZIP, as illustrated in Figure 8. Analysis for both Late 2022 and Early 2023 revealed common risk clusters across all three models, with no districts meeting the MLC criteria. However, the rank scores of districts varied among the models. Furthermore, these clusters demonstrated statistical significance based on the rank-based method, marking them as potential risk clusters for disease outbreaks and thus primary targets for surveillance. This consistency across models underscores the robustness of our risk cluster estimates. This comprehensive method of comparing results across different distribution-based models enriches our understanding of ASF outbreak dynamics. It distinctly showcases the strength of risk cluster identification derived from varied distributional assumptions and analytical methods.

In previous analyses (Figures 7, 8), it was assumed that intensities of surveillance were constant across all affected areas. However, considering the limited resources, it is plausible to adjust response intensities based on observed severity in different districts. Therefore, we designated surveillance intensity based on the concentration of discovered ASF-infected carcasses as a criterion for severity. We adjusted carcass counts accordingly and used these estimates to identify risk clusters, as depicted in Figure 9.

Surveillance zones for each period were delineated using the SDE method. The surveillance area was segmented into three zones based on sigma settings, with intensities assigned as strong, intermediate, and low (Figures 9A,B). We analyzed risk clusters and MLC with an observation error rate of 25%, as shown in Figures 9C,D, and found the results generally similar to those from analyses with uniform surveillance intensity at an observation error rate of 0% (Figures 7A,B). Even when the observation error rate was increased to 50%, hardly any significant differences were observed in the identified risk clusters (Figures 9E,F). Most districts with high carcass count and elevated risk levels were situated in areas of strong intensity (1-sigma districts), mitigating the impact of observation errors. As a result, the top-ranked risk clusters remained stable across all settings, demonstrating that the identification of risk clusters is reliable, even with considerable observation errors in districts with lower surveillance intensity.

Additionally, most of the lower-ranked risk clusters were located in districts with low intensity (3-sigma districts). The comparison of the bottom five risk clusters based on rank scores is detailed in Supplementary Table S2. Across settings with observation error rates of 0, 25, and 50%, some differences in rank scores were observed. However, during the Late 2022 period, the lower-ranked risk clusters were consistently identified across all settings. In Early 2023, four out of the five districts identified at a 0% observation error rate were also included in the bottom five risk clusters at the 25 and 50% error rates. This confirms that even significant observation errors lead to relatively minor differences in identifying lower-ranked risk clusters. Consequently, despite varying error levels, no significant differences were observed in the identification of risk clusters, underscoring the robustness of the risk identification process under different surveillance intensities and error conditions.

4 Discussion

The continuous nationwide spread of ASF has led to large-scale pig disposals, raising concerns within pig-farming communities and

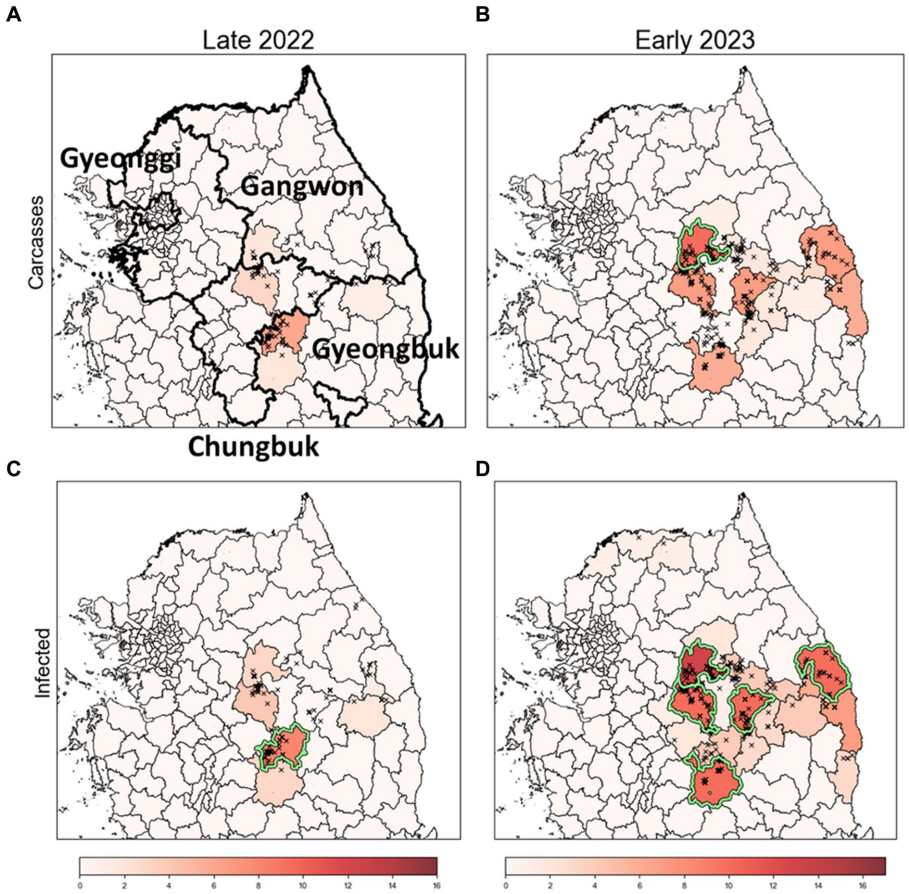

FIGURE 7
Identification of risk clusters and the most likely clusters for ASF using obsC and estI. **(A,B)** Risk clusters based on observed ASF-infected carcass data (obsC) for Late 2022 **(A)** and Early 2023 **(B)**. **(C,D)** Risk clusters based on estimated infection data (estI) for Late 2022 **(C)** and Early 2023 **(D)**. Districts marked in red indicate risk clusters, with deeper shades signifying higher rank scores. The districts with a light green colored border are identified as the most likely clusters (MLC).

imposing significant burdens on the livestock industry, public health, and the environment. The absence of vaccinations, effective treatment methods, and the risk of infection from ASF-infected wild animals and carcasses makes controlling and preventing ASF challenging. To mitigate these challenges, the Korean government has installed broad fences as a control measure against the spread of ASF (3), though their effectiveness is limited to certain regions (34).

Understanding the spatial distribution of the spread, as informed by previous incidence data, can improve the effectiveness of containment strategies. The prevalent practice of intensive livestock farming in the Republic of Korea (25), which minimizes contact between farmed and wild animals, underscores the pivotal role of wild animal movements in the spatial transmission of ASF. We analyzed surveillance data on the carcasses of wild boars infected with ASFV in the Republic of Korea from October 2019 to April 2023. Our study aimed to analyze the spatiotemporal distribution of ASF in the Republic of Korea and identify the risk clusters of ASF transmission. We aimed to develop a statistical modeling approach to understand the spreading patterns of ASF and identify risk outbreak areas, thereby identifying crucial regions for surveillance intensities.

Initially, we used the SDE method to quantify the annual spatial dispersion and directional trends of ASF outbreaks based on their geographic locations (Figure 6). Subsequently, we developed a GLM that incorporates the distance of observed districts from the center of the ellipse, along with seasonality and time as explanatory variables. Employing a Poisson distribution, this model calculated the average number of carcasses. Subsequently, we ranked the districts based on these calculations and compared these rankings with the actual carcass counts using a rank-based method to identify risk clusters. To more accurately reflect the real-time infection status, we implemented a comparative approach, analyzing risk cluster identification based on both estI and obsC (Figure 7). Furthermore, we enhanced the robustness of our findings by comparing results derived from Poisson, ZIP, and NB distributions to ensure that our findings were not confined by the assumptions of any single distribution model (Figure 8). Given the constraints of limited resources, it is possible to adjust response intensities based on the severity observed in different districts. In response to this, we estimated adjusted carcass counts accordingly and used these estimates to identify risk clusters (Figure 9). This comprehensive approach allowed us to identify risk clusters from various perspectives, thereby enhancing the robustness of the results.

Our study revealed several key findings regarding the spread of ASF in the Republic of Korea, highlighted by three main points. First,

TABLE 3 Comparison of top five risk clusters based on data for observed ASF-infected carcass counts (obsC) and estimated ASF-infected individuals (estI) with districts of high carcass counts.

Period	Districts with high carcass count	Risk clusters based on rank score using estI (Rank score)	Risk clusters based on rank score using obsC (Rank score)
Late 2022	A10. Mungyeong	A10. Mungyeong* (8)	A10. Mungyeong (6)
	A6. Chungju	A6. Chungju (4)	A6. Chungju (3)
	A2. Wonju	A2. Wonju (3)	A2. Wonju (2)
	A7. Danyang	A12. Sangju (3)	A12. Sangju (2)
	A9. Bonghwa	A9. Bonghwa (2)	A19. Bonghwa (1)
Early 2023	A2. Wonju	A2. Wonju* (13)	A2. Wonju* (10)
	A6. Chungju	A5. Samcheok* (10)	A5. Samcheok (7)
	A7. Danyang	A6. Chungju* (10)	A6. Chungju (7)
	A3. Yeongwol	A7. Danyang* (10)	A7. Danyang (7)
	A12. Sangju	A12. Sangju* (10)	A12. Sangju (6)

A1, A2, …, A12 denote the location information for each district, as indicated in Supplementary Figure S3. (Late 2022: September to December 2022, Early 2023: January to April 2023). (•) denotes the rank score. *Most likely cluster.

we noted both temporal and spatial variations in the ASF outbreaks. Our analysis showed that the number of ASF cases was approximately 3.82 times higher during the spring and winter seasons (HT season) compared to the summer and autumn seasons (LT season; Figure 4). This finding aligns with previous research (53) indicating that ASF occurrences tend to be more prevalent during relatively colder seasons in the Republic of Korea. By confirming temporal heterogeneity through the ADF test, we have statistically validated that the spread of ASF varies significantly across different seasons. Furthermore, spatial analysis indicated that 81.01% of ASF occurrences were concentrated in the Gyeonggi and Gangwon regions, highlighting significant spatial disparities between epidemic and non-epidemic districts (Figure 5). This emphasizes the impact of geographical factors on the spread of ASF. Second, we observed spatial dispersion trends between 2019 and 2020, initially showing a predominantly horizontal eastward trend, then shifting to a vertical southward dispersion in 2021 and 2022. This shift, along with the trend towards a dispersion ratio close to one by 2023, suggests a potential stabilization of ASF spread southward. The dispersion patterns correlated with the habitats of wild boars and major mountain ranges in the Republic of Korea, such as the Taebaek and Sobaek Mountains (54). This suggests that the spread of ASF is influenced by natural geography and the movement of wild boars. The observed stabilization from 2022 to 2023 is speculated to result from various control policies implemented in the Republic of Korea, including the installation of extensive fencing systems (30, 54). Third, we identified risk clusters for ASF outbreaks using statistical models, including the Poisson, NB, and ZIP models. Remarkably, the identified risk clusters aligned with the geographic locations of the Republic of Korea's major mountain ranges, as corroborated by spatial dispersion trends. Additionally, we estimated the number of infected individuals to aid in identifying risk clusters. While there were no significant differences in the districts designated as risk clusters, the number of districts classified as MLC differed. Furthermore, we compared the identification of risk clusters based on adjusted ASF-infected carcass counts across varying observation error rates (0, 25, 50%). The results confirmed that risk cluster identification exhibited no significant differences across all scenarios. The findings provide insights to enhance the effectiveness of surveillance strategies and control measures. Specifically, they suggest the potential for developing customized preventive strategies based on seasonal and regional variations, thereby improving the efficiency of resource allocation for disease control and providing evidence for targeted intensive control measures to effectively suppress the spread of ASF by identifying risk clusters. These insights underscore the utility of statistical models in enhancing preemptive actions such as wildlife population control, carcass removal, and the installation of extensive fencing, all of which are currently implemented in the country.

This study has several limitations. Due to the challenges in collecting real-time data on ASF-infected wild boars, our analysis was restricted to surveillance data derived from wild boar carcasses, as observed in previous studies (36, 37). This reliance on surveillance data may lead to potential risk underestimation compared to actual occurrences. Therefore, our approach focused on understanding dispersion trends and identifying potential risk clusters rather than predicting the exact number of carcasses in real time. Unlike previous studies that have largely focused on predicting risk clusters rather than estimating carcass numbers (53), our research incorporates environmental factors, such as forest area, distinguishes time intervals into HT and LT seasons, and analyzes risk clusters, marking a novel approach in the field.

Furthermore, the inability to determine the exact moment of infection based on the timing and location data of the carcasses limits precise outbreak analysis. Recognizing these constraints, previous research has concentrated on assessing the risk of infection. However, by applying the GLM and rank-based score methods, our study goes beyond merely identifying regions with a high number of carcasses by pinpointing areas with significantly higher occurrences than average, based on the discrepancy between actual data and model estimates. The application of various probability distributions, including Poisson, NB, and ZIP distributions, to identify risk clusters demonstrates the importance of a comprehensive analysis for understanding ASF dynamics. Additionally, we estimated ASF-infected cases from surveillance data and compared these with identified high risk and caution areas to verify underestimation due to risk estimation based on ASF-infected carcasses. This approach highlights the significance of identifying areas with significantly higher risk potential than expected, offering a nuanced understanding of ASF spread, and contributing to more effective disease management and prevention strategies.

Another point is the enhancement of efficiency and accuracy in identifying risk clusters through the application of artificial intelligence (AI) methods. In the previous study by Orusa et al. (55), the use of AI methods in conjunction with GIS and remote sensing technologies demonstrated the potential of geospatial deep learning AI to process complex datasets and provide actionable insights for wildlife disease monitoring and management. By leveraging AI, it is possible to develop more robust and adaptable disease management strategies that can respond to the evolving dynamics of diseases such as ASF. This integration enhances the efficiency and accuracy of identifying risk clusters, thereby improving the effectiveness of targeted surveillance and control measures. Such an approach will be instrumental in advancing our understanding of ASF dynamics and enhancing our ability to implement timely and effective interventions.

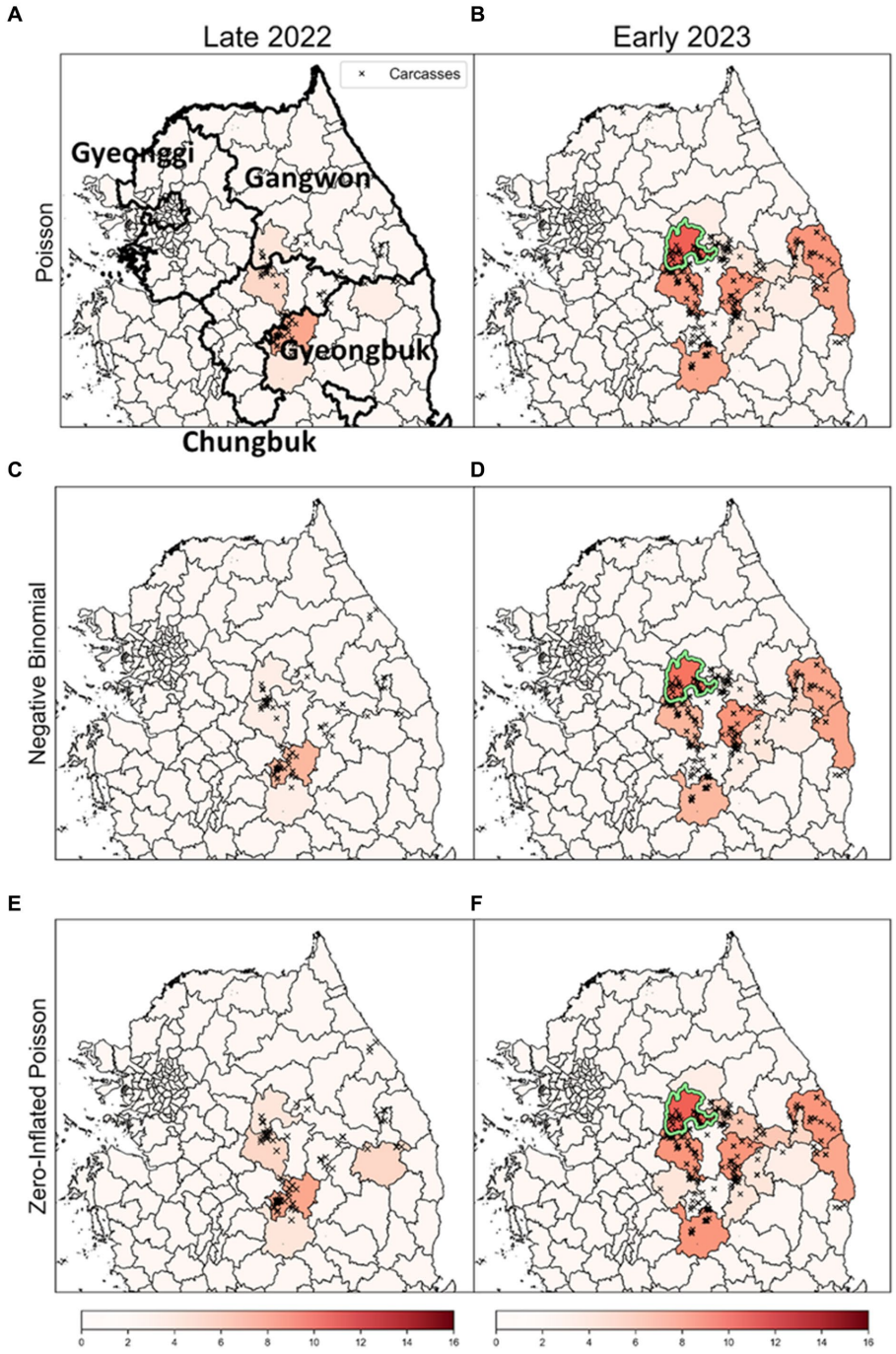

FIGURE 8
The results of a rank-based approach on different distributions. **(A,C,E)** show the results for the risk clusters from September to December 2022 (Late 2022), in row order according to each model. Similarly, **(B,D,F)** represent the estimates for the risk clusters from January to April 2023 (Early 2023), in row order according to each model. The colors of the dots signify monthly carcass counts. The districts with a light green colored border are identified as the most likely clusters (MLC).

Despite these limitations, the application of multiple statistical models and the integration of environmental factors, such as forest areas and seasonal variations, mark an alternative approach in the field. Our findings underscore the significant influence of temporal and spatial heterogeneity on the spread of ASF in the Republic of Korea, highlighting the intricate relationship between ASF dynamics, geographical features, and the role of wild boar habitats and movement patterns. Identifying risk clusters for targeted surveillance and control measures is crucial, contributing to more effective disease management and prevention strategies. This study offers insights into the strategic planning of surveillance and control measures, aiming for a more targeted approach for managing ASF outbreaks.

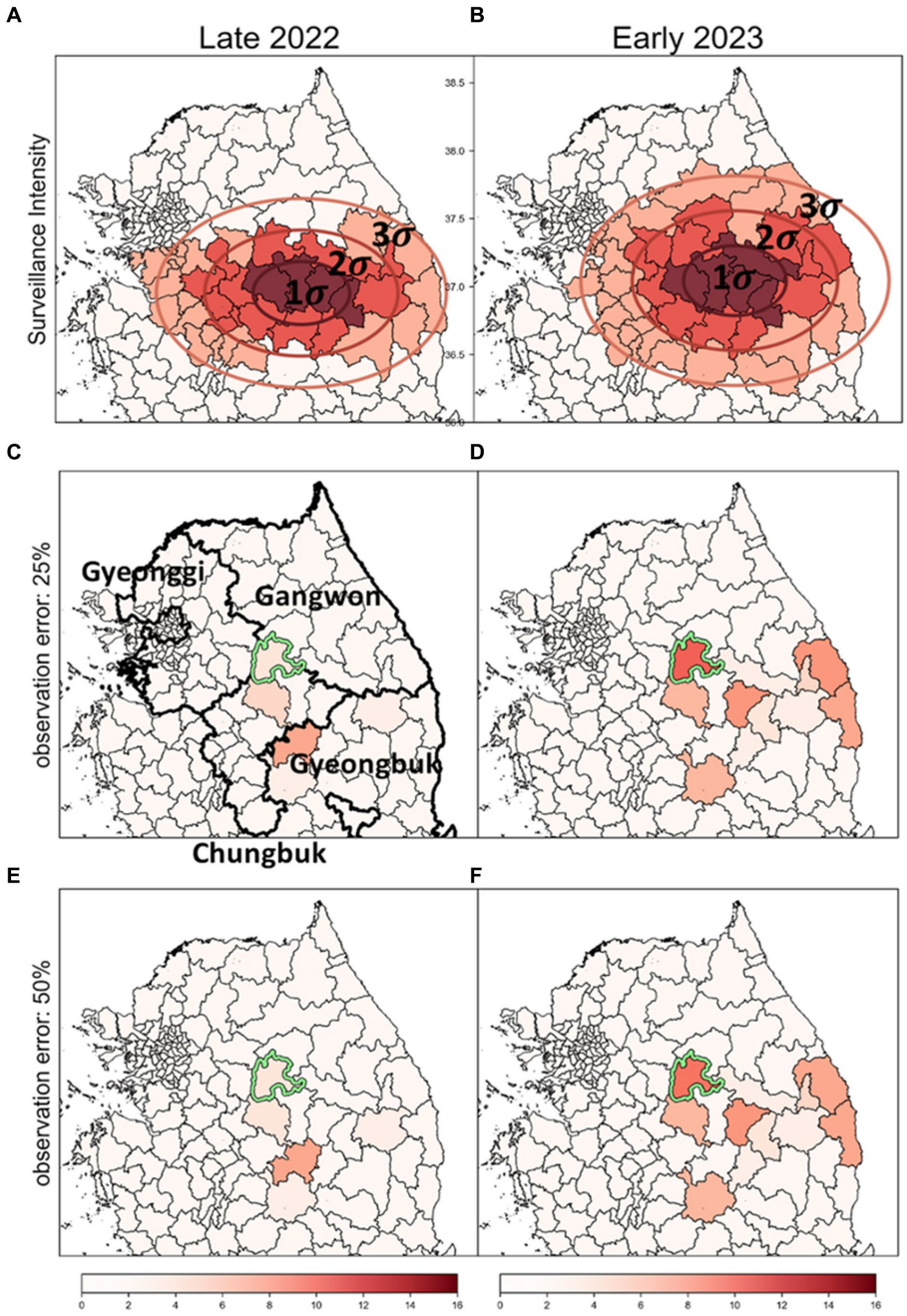

FIGURE 9
The impact of surveillance intensity and observation error on the identification of ASF risk cluster **(A,B)** Surveillance zones set up using SDE method; these zones are stratified into three levels of intensity: strong (1-sigma), intermediate (2-sigma), and low (3-sigma), to reflect the severity of ASF infection based on carcass counts. **(C,D)** Outcomes of the risk cluster when an observation error rate of 25% is applied. **(E,F)** Outcomes of the risk cluster when an observation error rate of 50% is applied. Districts marked in red indicate risk clusters, with deeper shades signifying higher rank scores. The districts with a light green colored border are identified as the most likely clusters (MLC).

5 Conclusion

This study analyzed the spatial and temporal heterogeneity of ASF in the Republic of Korea using surveillance data and revealed an annual southward propagation pattern. The angles of the SDE ellipse in 2021 and 2022, denoted as 109.5758 and 193.6745, respectively, indicate an annual southward propagation pattern. However, the ratio of the SDE ellipse in 2023, which was 0.9819, indicates that the southward movement was suppressed in 2023. This could be interpreted as an effect of measures such as the installation of extensive fences in certain areas during that year. We introduced a new statistical model that allowed us to predict the average monthly number of carcasses per district. We successfully identified risk clusters with significantly higher ranks based on observed ASF-infected carcasses compared to areas with high ranks based on the estimated ASF-infected carcasses. This study contributes significantly to the epidemiology and dynamics of animal infectious diseases by emphasizing the

importance of understanding spatially concentrated risk clusters. By providing crucial data for the efficient allocation of disease management and preventive measures, this study lays the foundation for improving ASF management strategies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

KK: Visualization, Validation, Formal analysis, Data curation, Writing – original draft, Methodology. JO: Writing – original draft, Visualization, Formal analysis, Data curation. YC: Writing – original draft, Validation, Methodology, Conceptualization. HL: Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization. CS: Writing – review & editing, Methodology, Visualization.

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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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1416862/full#supplementary-material

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African swine fever; insights into genomic aspects, reservoirs and transmission patterns of virus

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African swine fever is a hemorrhagic disease of pigs with high mortality rates. Since its first characterization in 1921, there has been sufficient information about African swine fever virus (ASFV) and related diseases. The virus has been found and maintained in the sylvatic cycle involving ticks and domestic and wild boars in affected regions. The ASFV is spread through direct and indirect contact with infected pigs, their products and carrier vectors especially *Ornithodoros* ticks. Severe economic losses and a decline in pig production have been observed in ASFV affected countries, particularly in sub-Saharan Africa and Europe. At the end of 2018, the ASFV adversely affected China, the world's leading pork-producer. Control strategies for the disease remained challenging due to the unavailability of effective vaccines and the lack of successful therapeutic measures. However, considerable efforts have been made in recent years to understand the biology of the virus, surveillance and effective control measures. This review emphasizes and summarizes the current state of information regarding the knowledge of etiology, epidemiology, transmission, and vaccine-based control measures against ASFV.

KEYWORDS

African swine fever virus, pigs, ticks, recombinant vaccines, *Ornithodoros*

Introduction

African swine fever virus (ASFV) belongs to the family Asfarviridae is a double-stranded DNA virus that causes African swine fever in Suidae (1, 2). In its ancestral African habitat, the ASFV evolved approximately 300 years ago in its arthropod vector in a sylvatic cycle, specifically involving common warthogs (*Phacochoerus africanus*) and the soft tick (*Ornithodoros moubata*) (3). The ASFV is epidemic in nature, causing large-scale mortality in the infected pig population (4). Serious economic consequences accompany outbreaks of the disease and therefore require proactive surveillance and management (5). Horizontal transmission of ASFV occurs through the feeding of swill containing infectious pig meat, contaminated pig-related products, and competent vector

species, especially *Ornithodoros* ticks (6). Transmission of the ASFV occurs within warthog burrows, primarily between ticks and warthogs (7).

In the late 1800s, the swine industry became larger in Kenya under British colonization, which significantly contributed to the prevalence of ASF and its subsequent distribution in Africa (8). The virus spread out of Africa in two different instance. The first incursion occurred in 1957 in Portugal, where ASFV-infected and contaminated waste materials were thrown from airline to feed the pigs, leading to the virus spreading through Russia, Western Europe, the Caribbean and Brazil over three decades. However, except for Sardinia, the disease was eradicated from all these affected areas by the mid-1990s (9). In 2007, a second incursion occurred in Georgia, expanded to the Russian Federation and Eastern Europe, and then spread globally (3). Globally, China is the largest pork producing country that is adversely affected by ASFV. In Europe, successful precautionary measures were limited to the Czech Republic, Belgium, Sweden, and Germany. There are high risks of the ASFV introduction to the United States, which is the third largest pork producing country in the world after China and the European Union (10). In July 2021, the USDA confirmed the entry of ASF into the Dominican Republic which then reached Haiti by September, marking a considerable geographical invasion and emphasizing the risks of ASFV being introduced into mainland North America. Large-scale movements of humans and animals are the major threat to those countries, that are free from ASFV (11). The increase in global trade of various goods and animals within or outside the country, provides a favorable means in the transmission and expansion of ASFV (12). Reducing the risks of ASFV, it requires a global attention to limit further expansion of this havoc-transmitting agent. Comprehensive knowledge of ASFV is necessary to overcome and eradicate the disease; for this purpose, this review is designed to highlight the etiology, epidemiology, transmission sources, and current development in prevention and control measures against ASFV.

Microbiology of ASFV

Structure of the virus

The shape of ASFV is icosahedral, and has an average diameter of 200 nm. There are four concentric layers in the structure of ASFV, an outer hexagonal membrane acquired by originating through the cell plasma membrane. The capsid is the outermost layer of the virion. The internal core of the ASFV particle is formed by the central genome and contains nucleoids, which are coated by a thick capsid in a layer called the core-shell (Figure 1) (13). For immunological interactions with the host, viral genomes encode genes essential for the replication of virus assembly (1). The virus replication process occurs mainly in the cytoplasm of infected macrophages and monocytes; however, it has also been observed in the nucleus at the early stages of infection (14). The ASFV is enveloped by a two-membraned collapsed cisterna, which is originates from the endoplasmic reticulum (15). Four classes of viral genes have been identified comprising immediate-early, early, intermediate, and late transcripts. Viral genes are usually express before and after the onset of DNA replication (16, 17). Enzymes are required for the ASFV replication and are expressed after the entry of the virus into the cytoplasm (18).

Genomic aspects

The ASFV genome is a single linear molecule of double stranded DNA having covalently closed double stranded DNA (19, 20). The P72 is the primary capsid protein, encoded by the *B646L* gene. The genotyping and sequencing of ASFV depend on a variable region of the *B646L* gene within the respective C-terminal of the gene. The ASFV genome ranges from 170 to 190 kilobases (kb) encoding 150–200 viral proteins, and has predicted open reading frames ranging from 151 to 186 kb (21). The ASFV has low mutation rate due to the accurate DNA proofreading.

The sequencing of the ASFV genome has resulted in the generation of several ASFV genomes of viral and low pathogenic isolates. Genomic analysis of ASFV has provided important information on structure, variation, and precise phylogenetic reconstruction (22). The *BA71V*, first strain of ASFV sequenced in 1995 (23), has been used as a comparative model for ASFV, which accumulates large scale information related to its biochemical, morphogenetic and genetic behavior (24). The genotyping and sequencing of ASFV depend on a variable region of the *B646L* gene within the respective C-terminal of the gene (25). Different virulent and pathogenic sequences have been identified and characterized from different origins and deposited in GenBank for information and experimentation (22). Until to 2018, 19 full-length sequences of ASFV were available which were generated using the Sanger sequencing; the number of sequences increased to 114 in October 2021 (27, 26). The multigene family (MGF) are responsible for the variation of ASFV genomes (1). The deletion and insertion of copied regions occur within these five MGF genes, suggesting the role of MGF in generating antigenic variability, thus helping the virus to evade host immune response (28). Based on the *B646L* gene, ASFV is categorized and divided into 24 different genotypes, the p72 capsid protein is coded by the *B646L* gene of ASFV (14).

Different isolates of ASFV can induce variable severity of infection. Although mild and moderate complications are caused by mutated strains (1). Pathogenic and virulent strains of ASFV are responsible for mild or severe infection accompanied by symptoms like hemorrhages in the skin and internal organs with a high fever and at the final stage causes death. Sudden deaths of animals can lead to 90% of fatalities (29). It has been suggested that the range of host and virulence of ASFV depend on the members of MGF 360 and 505. Some reports have demonstrated that the removal of eight genes from family 360 and two genes from family 505 affects its virulence to infect macrophages (30). The virulence of ASFV is due to four genes: the thymidine kinase coding genes, 9GL (B119L in BA71V), United Kingdom gene (DP96R in BA71V) and NL-S gene (DP71L in BA71V) (31). Therefore, further studies are necessary to understand the viral genome of ASFV as well as the genes associated with its pathogenicity: such genes will be supportive of the development for efficient diagnosis and treatment.

Genome sequences highlight variations between viruses in terms of insertions, deletions, or mutations. Comparative genomic analysis facilitates evolutionary studies. In this context, the first detected variation was the insertion of tandem repeat sequences (TRS), of the 10-nucleotide "TATATAGGAA" present between *173R* and *1,329*. This insertion has been recognized for the first time in ASFV infecting wild boar in Poland and Lithuania in 2014, and now it is considered a new sub-genotype marker (14).

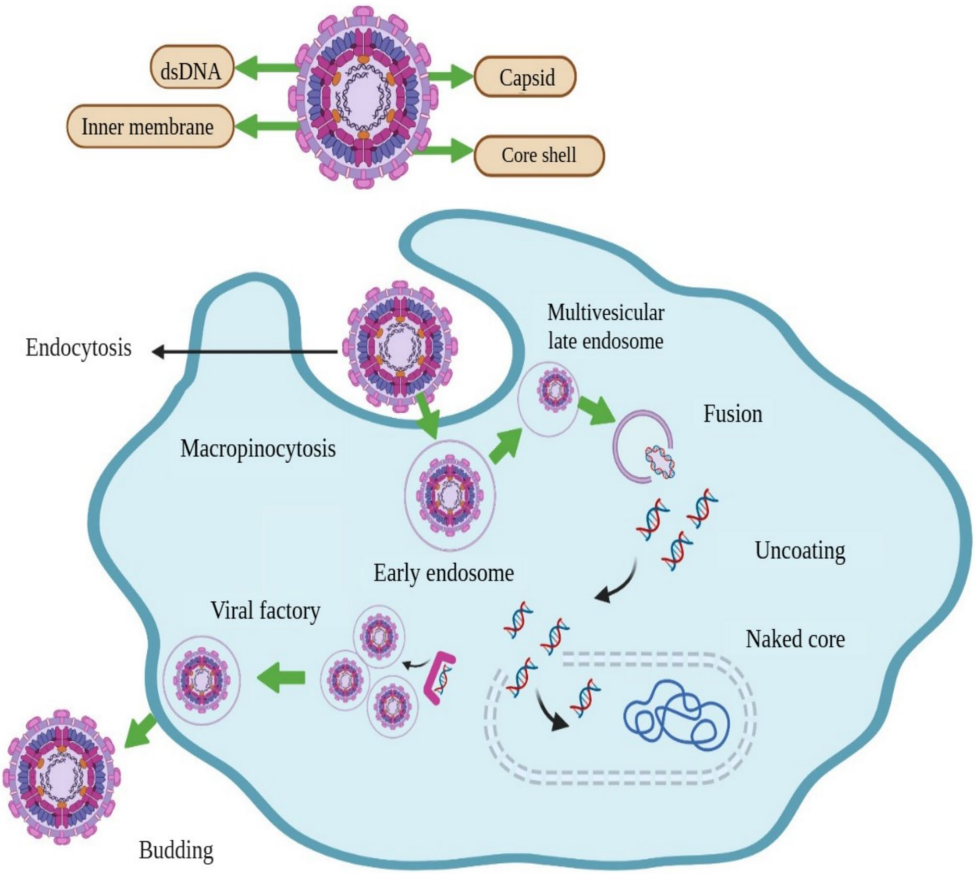

FIGURE 1
Structure and schematic presentation of African swine fever virus and mechanism of its entry into host cell, replication, and release.

In China, comparison of identified sequences of two strains, DB/LN/2018 and Pig/HLJ/2018 indicated mutations due to the insertion and deletion of nucleotides at multiple positions in the genome (32). The length of the genome sequence of both viruses was found to be 189,404 bp, very similar to the genomes of PoL/2017, GA/2007/ and ASFV-SY18. A comparison of DB/LN/2018 and Pig/HLJ/2018 with GA/2007 revealed 16 inserted nucleotides and 9 deleted nucleotides at 15 and 5 positions, respectively. In the PoL/2017 viruses three deletions appeared, whereas no insertion or deletion is present in ASFY-SY18. Furthermore, five mutations observed in the ORFs of Pig/HLJ/2018 and DB/LN/2018, ASFV-SY18 and PoL/2017 strain. However, these mutations were not observed in the GA/2007 genome. Due to mutations in ASFV, as a result of insertion and deletion of nucleotides, it is confirmed that changes and alterations occur in ORFs. Although the comparisons with the database provided clues about genes that may modulate the virus-host relationship. It remains to be examined how these alterations occur and affect the pathogenic properties of the ORFs of ASFV.

Epidemiological profiles of ASFV

Warthogs and domestic pigs are the main reservoirs for the ASFV. It is known from the last 10 years' data on ASF in the global context that the country will be potentially at risk where pigs are commercially as well as having wild reservoirs. Ticks are responsible for the transmission of ASFV and act as "reservoirs." Infected animals blood has the highest concentration of ASFV; therefore, virus transmission occurs through direct and close interaction with infected animals. Use of infected pork products and fomites or contact with them and the mechanical vectors, e.g., biting flies, may also aid in the transmission of the ASFV to the hosts which are uninfected from ASFV (33).

The ASF is not zoonotic; there are only reports of animal infections and transmission; the epidemiology of ASF reflects the circulation of the virus within animal and arthropod reservoirs. The prevalence of the disease varies from region to region due to epidemiological differences. Virus epidemics or outbreak situations have been observed based on the geographical conditions of the associated area. Large-scale outbreaks of ASFV usually occur between 2007 (China, Thailand) and 2009 (Vietnam) (34).

Reservoirs of the ASFV

Although pigs are the most frequent reservoirs of ASFV, they exist in the sylvatic cycle between arthropod vectors specifically soft ticks, *O. moubata* and wild Suidae especially warthogs (35). Warthog burrows are the habitat of these ticks, where virus transmission occurs between warthogs and ticks (2). Recently,

ASFV has been shown to grow within leeches (36), suggesting the participation of these hosts in the environmental perseverance of the virus (37).

Role of Suidae

Members of the pig family known to be susceptible to ASFV include domestic pigs and wild boars (*Sus scrofa*), bush pigs (*Potamochoerus larvatus* and *Potamochoerus porcus*), warthogs (*Phacochoerus* spp.) and giant forest hogs (*Hylochoerus* spp.) (7, 38. Since wild boars and domestic pigs are the main reservoirs responsible for disease outbreaks, the prevention and control of infected pig herds are the main issues (39). Wild boars and domestic pigs show various clinical signs, from acute to chronic (40). Although death is the first indication of the disease in a per-acute form, loss of appetite, depression, cutaneous hyperaemia and elevated body temperature (> 41°C) are other clinical manifestations associated with the per-acute form. The acute form of the disease is typically characterized by pulmonary edema, respiratory distress, abortion in pregnant females, nasal and conjunctival discharge, skin hemorrhages, splenomegaly, extensive necrosis and high mortalities (41). For the first time, the chronic form of the disease was reported in the Iberian Peninsula. The chronic form of ASFV has also been evidenced in experimental inoculation of animals with European isolates (14). Wild boar shows the same signs as domesticated pigs; however, no signs are observed before death in virulent strains. In warthogs and bush pigs, ASF is most frequently asymptomatic (42). It has been observed that survivors of ASFV may not only play a key role in ASFV perseverance in endemic regions but also contribute to periodic incidences, outbreaks, and invasions of ASFV to uninfected animals in the disease free zone (43).

Role of ticks

Following the classical epidemiological patterns of infectious diseases, ASFV circulates between animals and blood sucking arthropods. Hematophagy has been considered as a critical factor for the transmission and acquisition of ASFV by arthropods. Like other vector-borne diseases, the presence of an arthropod and blood sucking vector is important for the invasion and transmission of ASFV from the reservoir to healthy animals (44). An important aspect of the epizootic cycle of ASFV is the specificity of the vector. Among ticks, soft ticks are known competent vectors for ASFV. This pathogen has been isolated from 20 species of soft ticks and many other hematophagous arthropods, including competent louse and flies (Table 1). Even leeches have been considered susceptible to ASFV. Moreover, leeches were also able to transfer ASFV to experimental animals (36). Viral DNA has also been detected in hard ticks (*Dermacentor reticulatus* and *Ixodes ricinus*) collected from the bodies of dead ASFV positive wild boars and also in flies (54).

Ornithodoros ticks play a major role in the transmission of ASFV. All stages of their development can be easily infected with the virus, in the blood meal when the virus is taken from an infected pig. However, under experimental conditions, not all ticks that feed on infected pigs or artificial membranes become infected. For instance, in the case of the *Ornithodoros erraticus* infection model, an infection rate of ticks of 83.1% (pig-feeding ticks) and 53.4% (membrane-feeding ticks) infection rate of ticks has been observed (48).

TABLE 1 Arthropods in which African swine fever virus has been detected.

Tick species	References
D. reticulatus *I. ricinus* *A. americanum* *A. mixtum*	(45)
O. porcinus	(46)
O. erraticus	(47, 48)
O. moubata	(49)
O. coriaceus *O. parkeri* *O. tunicate* *O. puertoricensis*	(50)
Other arthropods *Muscadomestica* (House fly)	(36, 51)
Hirudomedicinalis (leech)	(51)
Drosophila spp. (Fruit fly)	(51)
Culicidae spp. (Mosquitoes) *Haematopinussuis* (Swine lice) *Stomoxyscalcitrans* (Stable fly)	(52, 53)

Localization within tick

Rock (55) performed the first experiment on the localization of ASFV in, *Ornithodoros porcinus*. Initial replication of ASFV was observed in hemocytes (types I and II), epithelium of the midget, phagocytic cell, connective tissue, salivary gland, coxal gland and reproductive tissue, which were the secondary sites of virus replication. Similarly, the highest viral titers were detected in salivary glands and reproductive tissue after 91 days of infection (28).

Survival of ASFV in ticks

It is known that ASFV once infects the tick, is capable of remaining viable within the tick's body for a long period between 23 and 239 days, depending the tick species (56). Much longer survival has also been reported: 3 years in *O. moubata*, 5 years in *O. erraticus* and 502 days in *Ornithodoros coriaceus* (29, 50, 57). The data suggest that without contact with the swine population, the ASFV can survive within the tick population, therefore, making an alarming source of reinfection.

Transstadial and transovarial transmission

Ticks can transmit infectious agents, retained within their body, to their next stage (transstadial) or the next generation (transovarial transmission). Similarly, soft ticks are believed to transmit ASFV transstadially, as well as transovarially through sexual contact, and directly to susceptible animals (29, 58, 59). However, in the case of

transstadial transmission, the transmission rate of ASFV has been found to decrease with each molt (60).

Pig-to-pig transmission

Direct contact with infectious pigs has been established to be an effective mechanism of disease transmission. The domestic pig can transmit the virus through nasal fluid, secretion, and in excretion through urine. In a recent study, healthy pigs housed together with infected ones became infected after one to 9 days after exposure. Delayed infectivity of healthy pigs was also observed in those separated from infected ones (14). During the fights, environmental contamination occurs due to the shedding of the blood from the wounds of infected pigs or from fecal contamination by infected Suidae (38). The possibility of a "carrier" state persists in pigs as well as in other Suidae. In the Netherlands, carrier pigs recovered from an acute ASFV infection with the lower pathogenicity strain were found to transfer the disease by direct contact with the uninfected pig population. It is believed that the overcoming and disappearance of clinical signs and symptoms occur, and after a month the infected pigs shed the virus. However, the effect on transmission will depend on the survival and duration of the carrier status of the pig (61).

Transmission through the ingestion of contaminated feed

Direct contact with the environment or infected pigs can introduce the virus into their bodies through mucous membranes. However, some animals are infected via ingestion of infected food. The presence of ASFV has been confirmed in pork products such as pig fat, skin, meat, and other pig products used for different purposes (62). Similarly, seed contamination and fresh grass containing wild boars secretions are the key risks and threat for the transmission (63). Therefore, these sources should also be considered to prevent transmission to naive pigs.

Wild boar-to-pig transmission

For ASFV transmissions, recent research has confirmed that wild boars act as a susceptible host for the transmission to domestic pigs. In domestic pigs, ASFV can develop nonspecific signs and symptoms (63). Several fields studies have confirmed the spread of ASFV from wild boars to domestic pigs. In this context, a study conducted in Russia detected several cases of ASFV primarily in wild boars before being observed in domestic pigs, and the death of wild boars caused by ASF was observed in the vicinity of ASF-affected farms (64). Similarly, the housing of susceptible pigs with ASFV-infected wild boars became infectious after 6 to 12 days post-exposure (65). The European Commission tasked the European Food Safety Authority (EFSA) to study and review the evolutionary ability and tendency of matrices, including arable crops, vegetables, wood chips, sawdust, hay, straw and other related agents that are the threats of transmitting ASFV (66).

Although ASFV is likely to transmit from wild boar to domestic pigs, long-range invasions of ASFV are mostly caused by anthropogenic activities such as improper disposal of carcasses of infected wild boars or infected pigs, disposed of by hunters and farmers. The sedentary nature of wild boars is considered the basic reason behind this issue. Generally, wild boars spend much time seated, up to 100 kilometers in 6 months; therefore, mostly they cannot cross long distances (67). Transmission of ASFV occurs during the migration of an adult male or female for reproduction purposes, the life span of the disease is short in this case ranging from five to 7 days (63). We assume that long-range ASFV incursions are not associated with wild boars; however, one cannot ignore their role in the transmission of ASFV to domestic pigs in close contact.

Movement of virus through fomites

Recent experiments have suggested that the ASFV can remain in the blood, feces and urine of infected pigs. As ASFV can easily contaminate the environment, therefore, anything that is contaminated may act as a virus source (68). An example of this type of incidence occurred in Europe, where the disease was introduced by ships containing ASFV-contaminated kitchen and catering waste used to feed pigs near the surrounding areas. Subsequently, the disease spread to the Caucasian region, the countries of the European Union (69). However, fomites are usually considered equipment, clothing, bedding, footwear, or transport which is contaminated and whereby the virus can be moved to a new area (70). From the observation of these experimental procedures, the transfer and movements of ASFV with fomites should be considered as a possible way for ASFV to spread to virus-free areas.

Anthropogenic factors

Human activities are important risk factors for ASFV transmission (26). Anthropogenic activities responsible for virus transmission include legal and illegal transport of pigs and pig's products, insufficient biosecurity measures for pig holdings and noncompliance with hunting restrictions and control strategies during and after the ASFV outbreaks (71). The primary cause of ASFV transmission is anthropogenic activities that cause long-distance transmission events and the introduction of pig farms (72). In China an initial outbreak of ASFV was linked to the feeding of pigs with contaminated table scraps (73), and in Vietnam contaminated pork products were likely responsible for the first outbreak (74). In Asia, anthropogenic activities have played a key role in maintenance and transmission (74). Targeted interventions and advanced biosecurity measures are necessary to eradicate the transmission of ASFV due to human activities.

African swine fever in Asia

In Asia, ASFV is more prevalent, following a pathway from northeast to southeast. In these countries, the transmission of ASFV is favored by compromised safety measures related to human activities including the transport of infected and contaminated fomites and pig products (75).

The introduction of ASFV in Asia and especially the invasion of China had a drastic impact on the pig industry (76). Despite these efforts, the virus has persistently crossed international borders rapidly. Subsequent introductions have occurred in Cambodia, Bhutan, Malaysia, India, China, Indonesia, Mongolia, Thailand, North Korea, South Korea, Myanmar, Vietnam, East Russia, Timor Leste, the Philippines, and Hong Kong (Figure 2) (3). In 2019, ASFV outbreak reported in Mongolia was confirmed by the Organization for Animal Health or Office International des Epizooties (OIE) (77). Many of these countries are characterized by rural pig farms and small-holder operations, making the resulting outbreaks particularly challenging to monitor and manage effectively. The dynamic nature of the virus's spread necessitates ongoing scientific efforts to understand and address the complexities of ASFV transmission within diverse agricultural and economic contexts (26).

African swine fever in China

The rapid development of the Chinese economy has brought about significant changes in food consumption patterns. Similarly, demands for meat and meat products, especially pork, have shown a constant increase over the last few years (78). Taking this advantage, the swine sector of the country has integrated production systems to become an industry. Three types of pig farming have been practiced based on the number of pigs produced; small, medium and large farms. Farms close to large cities have been shifted from backyards to modern intensive farms (34).

In China, the first outbreak of ASFV was reported on August 3, 2018, when 400 pigs on a farm near Shenyang City in the north-eastern Liaoning province, developed acute clinical disease after consuming table scraps. The mortality rate was 100%, leading to abandonment of the farm. Subsequently, similar cases were observed on nearby farms (73). At the end of 2019, a total of 33 outbreaks were reported in eight provinces of the country, ASF has led to the deaths of more than 100,000 pigs with an estimated loss of USD 111.2 billion (79, 80). Without the availability of effective control measures, including vaccination, the resumption of production will be problematic. As a result, in China, pig production was reduced by 40% in 2019 compared to 2018. Similarly, the price of pork was doubled in 2019 (81). Therefore, the spread of this disease has posed threats to the large population of domestic pigs and wild boars in China, as well as in neighboring countries.

A strategy to control the outbreak has been developed by Chinese authorities (Ministry of Agriculture and Rural Affairs; MARA) and implemented soon after the emergence of the first outbreak. Culling all pigs within 3 km of the infected area and capturing all infected pigs, their disposals, and contaminants became mandatory. For the prevention and control of the ASFV outbreak, the Ministry of Agriculture and Rural Affairs has taken several preventive measures to control the outbreaks. Several preventive biosecurity measures were implemented including restricted pig movement, complete biosecurity protocols both inside and outside of pig farms, and systematic monitoring and recycling of pig products and waste materials. Quarantine measures have been enforced on farms, and high temperatures were applied for the treatment of feed and other waste

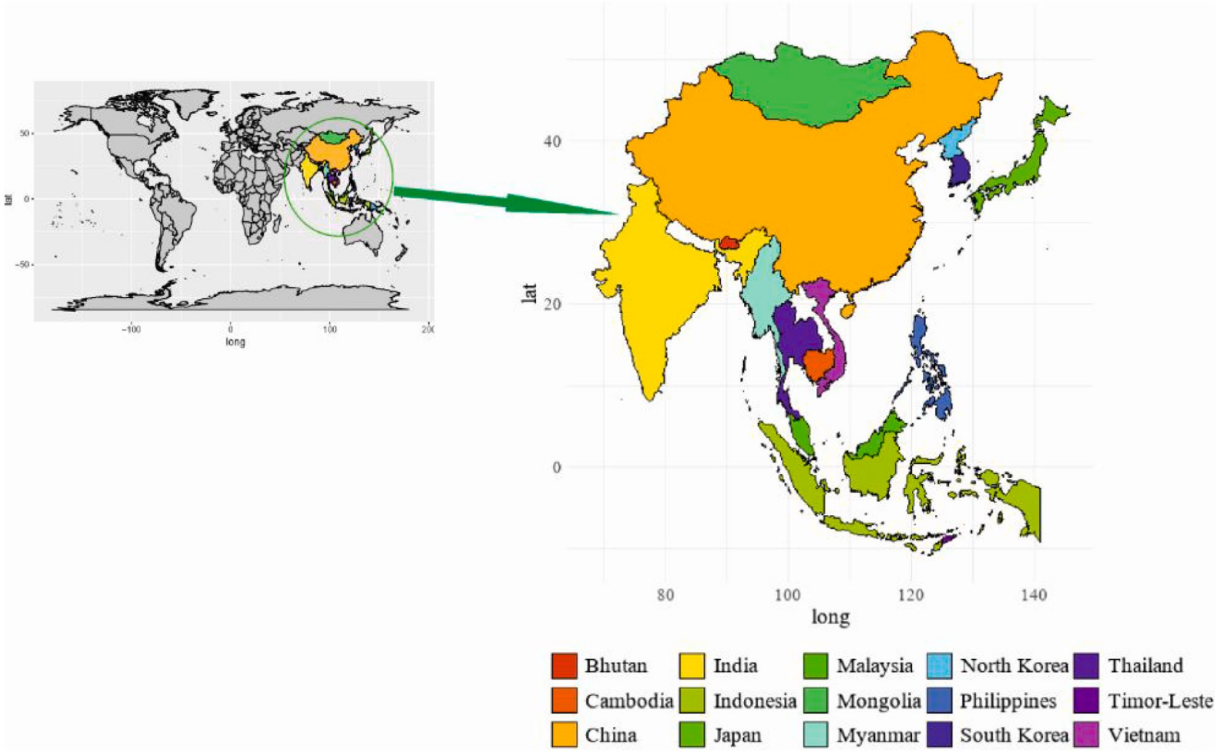

FIGURE 2
Geographical distribution of ASF in Asia.

materials of pigs (82). Despite these measurements, the epidemiological status in China become worse after the occurrence of ASFV new cases (83). The alarming spread and expansion of ASFV necessitates a dynamic scientific approach to control ASFV in various regions (84).

African swine fever in Africa

In Africa, ASFV evolved through a sylvatic cycle between the soft tick *O. moubata* and warthogs (85). From 1989 to 2017, 5,134 ASFV outbreaks were recorded with 88.5% occurring in the domestic pig population and wild Suidae (33). The expansion and transmission of ASFV to new areas occurs due to the movement of infected pigs into ASFV free-areas, while warthogs and wild suids also play a major role in its transmission and propagation in Africa (9, 64). In Africa, the spread of ASFV to new areas beyond its endemic region causes severe losses (33). Outbreaks arise from transmission between domestic pigs and the sylvatic cycle, with warthogs being translocated into the southern part of the continent over the past four decades (7). In 2017, ASF outbreaks had been reported in Cabo Verde, Côte d'Ivoire, Burundi, the Central African Republic, Chad, Kenya, Madagascar, Mali, Mozambique, Namibia, Niger, Sierra Leone, South Africa, Zambia, Zimbabwe, Tanzania, Sahara, Zanzibar, Malawi, Angola, Botswana, Ethiopia, Djibouti, Liberia, Senegal, Mauritania, Morocco, Mali, and Tunisia [(Figure 3; (86)]. The rapid expansion of the African population has demanded an increase in meat production. This has a direct impact on the pig population, which constitutes approximately 5% of the global pig population (33, 87). Some African countries, namely South Africa, Madagascar, Mozambique, Namibia, and Nigeria, reported one to multiple outbreaks. Likewise, the unquantified presence of the disease has also been reported in countries like Cameroon and Cape Verde (33, 85). At the end of 2017, 69 cases of ASFV were confirmed in which warthogs act as a source of the outbreak that occurred in Kenya, Namibia, Botswana, Zambia, Zimbabwe, South Africa and Tanzania (33). Restriction of wild pigs to conservation areas has reduced their likely role in the epidemiology of ASFV (75).

African swine fever in Europe

In Europe, the past 6 years have seen the introduction of ASFV to Belgium, Bulgaria, the Czech Republic, France, Germany, Greece, Hungary, Italy, Lithuania, Malta, Moldova, the Netherlands, North Macedonia, Poland, Romania, Serbia, Slovakia, Spain, Sweden, Georgia, Portugal, Latvia, Estonia and Romania (Figure 4) (88). Recently, the Czech Republic and Belgium, in which domestic pigs were not infected, now appear to have eradicated ASFV via biosecurity measures. Elsewhere (including Bulgaria, Hungary, Poland, Romania, and Slovakia in particular), the virus generally appears to be beginning ground, with numerous outbreaks, especially on smallholder farms (86). Similarly, the introduction of ASFV in northeast Lithuania resulted in the death of more than 20,000 pigs (14). Epidemiological investigations have revealed some details of the ASFV transmission patterns unique to these countries (e.g., Poland, where wild boar infections are dominant, versus Romania, where domestic outbreaks are more common) and have also identified the apparent evolution of lower-virulence ASFV strains in Estonia and Latvia (89). The ASFV genotype I was restricted to the African continent from its first

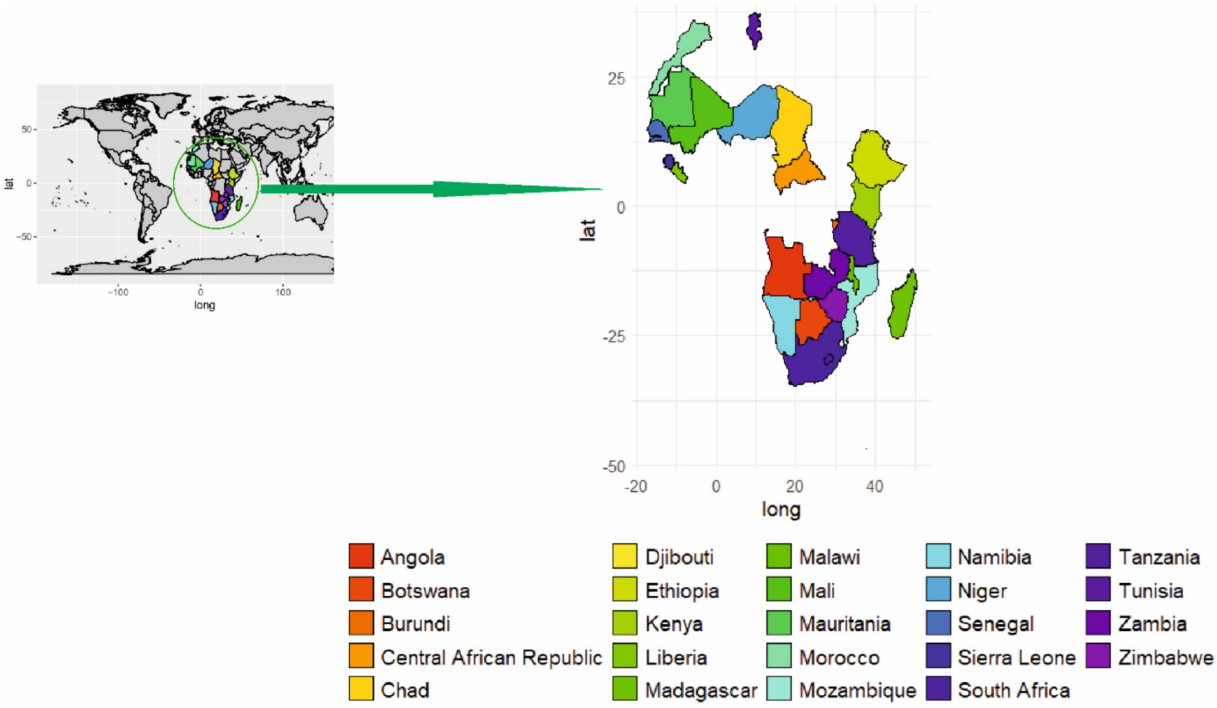

FIGURE 3
Geographical distribution of ASF in Africa.

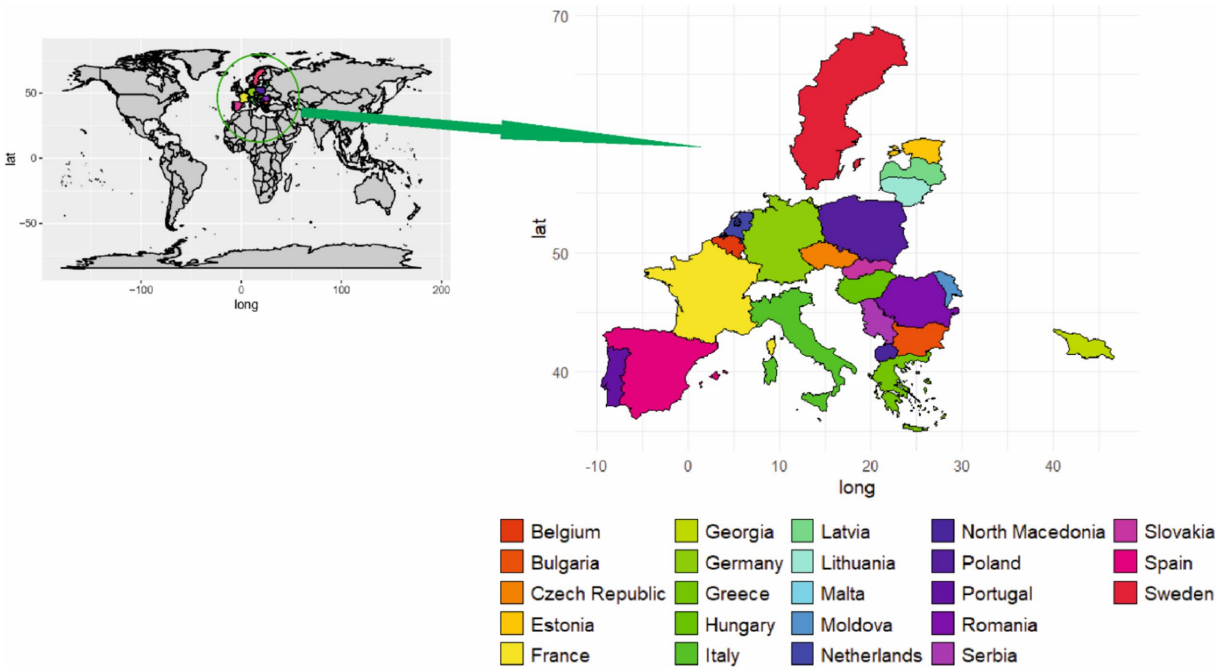

FIGURE 4
Geographical distribution of ASF in Europe.

recognition in 1920 until 1957, when the outbreak was reported in Portugal. Spain was the first European country to report ASFV cases, followed by Italy, France, Malta, Belgium, and the Netherlands (28, 90). Pork meat imported directly from Spain was thought to be the source of the first ASFV case in Belgium in 1985. The first outbreak of ASF resulted in the slaughter of 34,000 pigs housed on 60 holdings (29). The pig population in the Netherlands was severely affected from 1960 to 1995 due to ASF (29, 91). Although ASF was successfully eradicated from most European countries by 1995, a notable exception was the Mediterranean island of Sardinia (Italy). The main factors that were responsible for the persistence of the disease in that area were the keeping of more than 70% of the pig population in extensive systems and backyard farms, combined with the proximity of wild boars (92).

The lack of consistency in ASF contingency plans and preventive measures resulted in the second spread of ASFV in European countries (29). In this context, the first case was recognized in Georgia in 2007,followed by numerous outbreaks of domestic pigs and wild boars (28, 29, 64, 90). The specific origin of the virus responsible for the outbreak is still unknown; however, the virus genotype has been linked with that in Madagascar and Mozambique (90). The disease spread from Georgia to native European countries and was recently reported in Latvia, Estonia, Hungary, and the Netherlands (90). Not only in western Europe, the pig population was also severely affected in eastern Europe. Poland, a country where 66% of the pig population is kept on small farms, reported the first case of ASF in 2014 (90, 93). Since the first outbreak in Poland, authorities have reported several outbreaks, with a total of 5,333 cases of the disease being confirmed in wild boars (29).

Since the first appearance of ASF in Europe in (1960), it took 30 years to successfully eradicate the virus from affected countries (94, 95). The second spread of the disease (2007), contributed to virus migration in nearby regions, as well as a high probability of outbreaks in neighboring countries (90). Efforts to control disease in Europe have not been successful. Impediments to the development of successful eradication programs include low biosecurity (human factor), free-ranging wild boar populations, and a high prevalence of the virus in surrounding bordering countries (90). Low or non-existent biosecurity measures at small-scale pig holdings increase the risk of introducing viruses on the farm. Ticks, wild boar populations, and the illegal trade of infected meat products are the other factors responsible for maintaining and circulating the virus within pig populations (57). Despite the challenges, the European authorities have implemented multifaceted preventive and hygiene procedures. In addition, government institutions are strongly convinced of achieving the eradication of disease in a short period of time.

African swine fever in the United States and its future assessment

The United States (United States) is believed to manage pig production under high biosecurity conditions. Economic losses due to ASFV introduction into the U. S are estimated in between $15 and $50 billion, depending on the disease spread in the feral swine population (96). Commercial swine production is a closed system from farrowing to slaughter as a means of reducing the risk of pathogen introduction (97). To limit cross contamination, transport vehicles, animal feed, personnel and other fomites are closely managed. Despite the high-profile biosecurity measures, transport equipment contributed to the spread of the porcine epidemic diarrhea virus (PEDV) in 2013 (59). This indicates that despite stringent biosecurity protocols, it can be difficult to control ASF. Keeping this

in mind, the US has substantially contributed to the implementation of a series of preventive measures designed for the importation of live animals and their products.

Prevention and control strategies

The escalating intensification of animal movements and product exchanges have increased the risk of ASF in new regions, particularly those developing trade ties with new eastern EU member states (98). Despite of this threat, despite efforts, there is no effective vaccine available for global eradication. The developing of an effective vaccine becomes crucial to controlling one of the major pig diseases in Africa. Such a vaccine would offer an alternative to animal slaughter, mitigating the spread of ASF in both Africa and Europe (99). Challenges to eradication efforts include free-range production systems, interactions with *Ornithodoros* ticks and/or wild suids, and endemicity involving asymptomatic carriers (Wamwatila et al., 2015) (100). Success relies highly on effective communication between all involved parties in an outbreak, including diagnostic laboratories, farmers, field and official veterinarians, disease crisis centers, and media participation. Implementing improvements in pig housing to minimize contact with ticks and wild animals have proven highly efficient in reducing infection to eradication levels and should not be overlooked (1). A cost–benefit analysis should determine whether contingency efforts would be directed toward control or eradication (101).

In the absence of a vaccine and considering the role of the sylvatic cycle in the epidemiology of southern and eastern Africa's disease, logical measures for ASF control include the physical separation of domestic pigs from wild hosts and treating pig premises with acaricides in areas where tick-infected by ASFV occurs (28). Control based on the physical separation between wildlife and pigs has been proven successful in controlling ASFV, even in animals from those regions where the virus circulates among infected warthog populations (7).

Control strategy based on genetically modified vaccines

Despite the implementation of strong biosecurity measures, it is a difficult task to control the spread of disease in the pig population. Control measures rely on prompt reporting, repeat testing, and culling infected and at-risk animals. Moreover, in underdeveloped countries these methods and their establishment are difficult to apply. As an alternative, researchers are now struggling to develop an effective vaccine against ASFV. For this purpose, several attempts have been made in recent decades. Different vaccine strategies such as DNA vaccines, adenovirus vector vaccines, subunit vaccines, and inactivated vaccines have been tested and proven to be unsuccessful (2). Similarly, extracts of infected cells, purified and inactivated virions, infected leukocyte blood supernatants, infected glutaraldehyde fixed macrophages, or infected alveolar macrophages have been used to produce immunity against ASFV (62). However, all of these attempts failed to produce desirable results. Meanwhile, it was observed that pigs infected with attenuated or virulent variants of ASFV may establish resistance to homologous virus challenge (102). These observations led scientists to develop an effective live attenuated virus by deleting genes not associated with ASFV replication. Virulent isolates of ASFV have been modified with deletions of genes to attenuate the virus. Keeping this in mind, the BeninΔDP148R virus was genetically modified by deleting the *DP148R* gene to isolate the virulent strain, Benin97/1. Deletion of the gene reduced the pathogenicity of the BeninΔDP148R virus in pigs. All the pigs immunized with the virus showed only mild transient clinical signs and survived infection. Moreover, high level of protection was observed against the parental virulent strain (103). The same level of safety and protection was observed after immunization of the pig with ASFV-G-ΔI177L. After immunization, the pig showed a strong and specific antibody response and low viremia titers (104) (Table 2).

Based on available knowledge, the use of genetically modified viruses is the most reasonable approach to establishing an effective ASFV vaccine. Genetic modification and deletion of one or more genes change the virus

TABLE 2 Genetically modified ASFV by deletion of genes and their effects in immunized pigs.

Isolate	Gene deleted	Protection against parental virus	References
ASFV-G	TK (thymidine kinase)	No	(105)
ASFV-G (2007)	9GL (B119L)	High	(106)
NH/P68	A238L, A224L, EP153R and A276R	Moderate	(14)
Benin 97/1	DP148R	High protection	(103)
ASFV-G	I177L	High	(104)
ASFV-G (2007)	9GL and UK	High	(107)
HLJ/18	MGF505-1R, MGF505-2R, MGF505-3R, MGF360-12 L, MGF360-13 L, MGF360-14 L and CD2v.	High	(81)
Benin 97/1	MGF360 and MGF530/505	High	(103)
ASFV-G (2007)	MGF505-1R, MGF360-12 L, MGF360-13 L, MGF360-14 L, MGF505-2R, and MGF505-3R	High	(106)
OUR T88/3	DP71L and DP96R	Moderate	(106)
ASFV-G (2010)	8DR (EP402R)	Failed to induced	(104)
ASFV-G	9GL/NL/UK	Failed to induced	(104)
ASFV-G (2007)	9GL and MGF	Failed to induce	(106)

from virulent to less virulent (104, 108). In domestic pigs ASAFV-G-Δ8DR is responsible for disease state. Pigs infected with the ancestral virus ASAFV-G-Δ8DR show the same viremia values. More attention is needed for the selection of targeted genes. Attenuated viruses of different genotypes should be tested to obtain strains that protect isolates circulating in different regions. Moreover, optimized targeted genes are used for safety standards. Similarly, the issue of the availability of a licensed cell line to grow live attenuated viruses for vaccine production needs to be resolved. For efficient control measures, immune responses induced by virus antigenic proteins are necessary to enhance the protection of infected animals (108). Therefore, the rational development of novel ASFV vaccines requires caution and more work to optimize commercial production.

Conclusion

The current situation of ASF signifies a constant risk to the livestock sector. Recent exploration and flourishing of ASFV have demonstrated the ability of the virus to spread over long distances. As a result, there is a tremendous decrease in both the production and farming of pigs. Furthermore, the implications of the trade related to ASFV in swine have severely affected the pork industry. Veterinary services need to perform rigorous surveillance in countries that consume pigs, as the inaccessibility to effective medication persists, leading to high mortality rates are the main reasons. Biosecurity measures are crucial to prevent the transmission of viruses. Inadequate biosecurity practices can create opportunities for the spread of viruses, which pose risks to human and animal health. Vaccines have given some favorable results; however, further investigation is required to prove them as the only choice to treat and control the disease.

Author contributions

BA: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. C-HW: Writing – original draft, Writing – review & editing. MK: Writing – original draft, Writing – review & editing. MN: Writing – original draft, Writing – review & editing. C-CC: Writing – original draft, Writing – review & editing. AA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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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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A rosin-functionalized plastic surface inactivates African swine fever virus

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African swine fever virus (ASFV) causes a severe hemorrhagic disease in pigs, leading to up to 100% case fatality. The virus May persist on solid surfaces for long periods; thus, fomites, such as contaminated clothing, footwear, farming tools, equipment, and transport vehicles, May contribute to the indirect transmission of the virus. Here, a plastic surface functionalized with tall oil rosin was tested against ASFV. The rosin-functionalized plastic reduced ASFV infectious virus titers by 1.3 $\log_{10}$ after 60 min of contact time and killed all detectable viruses after 120 min, leading to a ~ 6 $\log_{10}$ reduction. In contrast, the infectious virus titer of ASFV in contact with low-density polyethylene (LDPE) plastic reduced <1 $\log_{10}$ after 120 min. Transmission electron microscopy (TEM) showed significant morphological changes in the virus after 2 h of contact with the rosin-functionalized plastic surface, but no changes were observed with the LDPE plastic. The use of antiviral plastic in the farming sector could reduce the spread of ASFV through fomites and could thus be part of an integrated program to control ASFV.

KEYWORDS

African swine fever, ASFV, rosin, antiviral surface, plastic

1 Introduction

African swine fever (ASF) is a severe hemorrhagic disease in pigs, leading to up to 100% fatality in infected animals. The disease is caused by the ASFV, which is transmitted through direct contact with infected domestic or wild pigs, pig products, or fomites. Fomites are objects or materials that are likely to carry infection. This could include all objects and materials that come into contact with infected pigs, such as feeds, housing, clothes, footwear, equipment, and transport vehicles (1). In addition, in sub-Saharan Africa, there is evidence for the presence of a sylvatic cycle, where ASFV is transmitted between warthogs and soft ticks of the *Ornithodoros* genus (2). In most countries in Sub-Saharan Africa, the disease is endemic, and several genotypes May circulate in an area simultaneously (3). Outside of Africa, only genotypes I and II, as well as recombinants of these genotypes, have been detected. ASFV genotype II spread to the Caucasus region of Georgia in 2007 and has since spread to many other countries in Europe, Asia, Oceania, and the Caribbean (4). Thus, ASFV poses a threat to the pork industry globally. In the absence of treatment and globally available effective vaccines, biosecurity measures are essential to prevent the spread of the disease. Biosecurity measures include limiting the movement of animals, people, and materials/equipment between farms, using foot baths, providing farm-specific PPE, and disinfecting equipment and vehicles entering and leaving the farm (5).

ASFV is a large and complex double-stranded DNA virus, which consists of multiple concentric layers and has a diameter of approximately 260–300 nm. The outermost layer is the external envelope, which is derived from the cellular plasma membrane during the budding process by which the virus egresses from the cell. Interior to this, the virus has an icosahedral outer capsid with a diameter of approximately 200 nm. Underneath the outer capsid lies an inner membrane, which surrounds the core shell and the nucleoid. Both the intracellular virion, without the outer envelope, and the extracellular virion, with the outer envelope, are infective *in vitro* (6–9).

The ASF virus is inactivated by treatment at 60°C for 30 min but remains infectious at lower temperatures and when stored frozen over extended periods. It has been estimated that ASFV can remain infectious for 8.5 or 15.3 days at 4°C, respectively (10). Furthermore, viable viruses can remain on the tissues of pig carcasses for several months, especially at low temperatures (11). Some studies have investigated ASFV viral survival in feed, bedding materials, and soil. Stoain et al. (12) demonstrated that the half-life of ASFV varied from 9.6 to 14.2 days when viral survival was studied in nine different feed ingredients in conditions that simulated trans-Atlantic shipment. Field viruses could be isolated from bedding materials for some days to weeks at 4°C, with the longest survival in bark. However, in soil, the stability of ASFV was shown to vary from a few days up to several weeks (13, 14). In rivers, the water virus remained infectious when maintained at 4°C but was inactivated after 28 or 14 days when maintained at 15 or 21°C (15). In samples stored between +4 and + 23°C, the inactivation of the virus was faster in water, soil, and leaf litter compared to straw, hay, and grain. In a study by Petrini et al. (16), infectious ASFV was detected in dry-cured meat products (loin and pork belly) prepared from infected pigs even after 2 months from the beginning of the processing.

Coniferous resin acids are known to have antimicrobial properties against a range of pathogens, including Gram-positive bacteria, fungi, and enveloped viruses (17–21). Rosin is a by-product of the forest industry and contains resin acids. We found recently that rosin-functionalized plastic leached the active component to the surface of the plastic and very potently reduced the infectivity of SARS-CoV-2 and seasonal coronavirus (HCoV-OC43) (19). This study aimed to investigate whether rosin-functionalized plastic has antiviral activity against the structurally complex ASFV.

2 Materials and methods

2.1 Plastic material

Two types of plastic surfaces were used for our study: low-density polyethylene (standard LDPE) and rosin-functionalized plastic (PREXELENT®), generously provided by Premix Ltd. PREXELENT® is a functional plastic that is prepared by incorporating 10 wt-% of rosin into LDPE (patent WO2018229190A1). The plastic surfaces were 1 cm^2.

2.2 Surface antiviral test

To test the contact time required for ASFV to be inactivated by the rosin-functionalized plastic (PREXELENT®) or LDPE plastic, ASFV was added to the plastics in triplicate and incubated for 5, 10, 30, 60, 120, 240, or 480 min. Before starting, the plastics were sterilized by immersing them in 70% ethanol for 30 s and wiping them with sterile tissue paper. They were then left to dry out and transferred to 24-well plates with 6 mL sterile water between the wells to prevent drying out of the plastics and incubated at 37 °C overnight. On the day of the assay, a volume of 50 µL of ASFV_Kenya1033 was used for each replicate (corresponding to 1×10^6 HAD_{50}). ASFV_Kenya1033 is a genotype IX ASFV and was grown in primary porcine macrophages (22). A volume of 50 µL ASFV_Kenya1033 was added on top of triplicate pieces of each plastic for each incubation time (5, 10, 30, 60, 120, 240, and 480 min). After incubation at room temperature (20–25°C) for a set amount of time, 450 µL of complete RPMI [RPMI 1640 (Sigma Aldrich, UK), supplemented with 2 mM L-glutamine (Sigma Aldrich, UK), 10% fetal bovine serum, 100 UI/mL of penicillin (Sigma Aldrich), and 100 mg/mL of streptomycin (Sigma Aldrich, UK)] was added to each of the wells. The plates were rocked on a plate shaker for exactly 1 min, and the resulting media was collected and aliquoted for ASFV quantification. For DNA extraction and real-time quantitative PCR analysis (qPCR), a volume of 200 µL was aliquoted and the remainder volume was aliquoted for analysis by hemadsorption assay.

2.3 Quantitative determination of ASFV

The ASFV genome copy number in samples was determined by amplification of p72/B646L using a qPCR assay adapted from King et al. (23). DNA was extracted from 200 µL of the sample using the DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. The resulting DNA was used for the qPCR analysis with primer and probe sequences adapted to the ASFV_Kenya1033 strain. The primer sequences were P72-F (5'-CTGCTCACGGTATCAA TCTTATCGA-3′) and P72-R (5'-GATACCACAAGATCAGCCGT-3′), and the P72 probe was 5'-FAM-CCACGGGAGGAATACCAACCC AGCG-TAMRA-3′. Each reaction was conducted in duplicates in 20 µL of reaction mixture containing 6.5 µL of nuclease-free water, 10 µL of TaqMan Fast Advanced master mix (Applied Biosystems), 0.6 µL of forward primer (10 µM), 0.6 µL of reverse primer (10 µM), 0.3 µL of TaqMan probe (10 µM), and 2 µL of template DNA. The plasmid standard was diluted 10-fold from 10^9 to 10^1 copies/ well. Nuclease-free water was used as no template control.

The titer of infectious virus in samples was determined using a hemadsorption assay. The samples were 10-fold serial diluted in complete RPMI (see above), and the different dilutions were used to infect porcine pulmonary alveolar macrophages (PAMs) in 96-well plates using four replicates per dilution for each sample. The next day, red blood cells were added to the plates (1:200 diluted in PBS). The plates were examined for the presence of the ASFV characteristic rosettes using an inverted microscope on day 5. The titers of infectious viruses in the samples were calculated using the Reed and Muench method.

2.4 Preparation of virus for imaging

ASFV (genotype II ASFV grown in primary porcine macrophages; 1×10^6 HAD_{50}) was added onto the surface of the plastic surfaces that were placed in a well of a 12-well tissue culture plate and sealed. The

virus was added in 10 µL of the stock virus, and a 13 mm coverslip was added to ensure good contact for 2 h incubation time at room temperature. After 2 h, 500 µL of 10% formaldehyde fixative was added, which lifted the coverslip off. The coverslip was then taken gently away, and fixative incubation continued for 48 h at room temperature. The fixative solution containing detached viruses was transferred to tubes and fresh 10% formaldehyde fixative was added to cover the plastic surface. The plastic sheet and fixative containing detached viruses were placed in containers for shipment to Finland.

2.5 Imaging of virus using transmission electron microscopy

To concentrate the sample for electron microscopy, 350 µL of the media that was flushed from the rosin-functionalized plastic (PREXELENT®) and LDPE plastic was centrifuged gently using the Beckman Coulter Airfuge™ centrifuge (20 psi, 30 min, A-95 rotor). The pelleted virus was gently dissolved in a small volume of 10–15 µL of the supernatant left in the tube after pelleting.

For the TEM sample preparation, 5 µL of the pelleted virus was applied to glow-discharged Butvar-coated copper grids (EMS/SC7620 mini-sputter coater) for 15 s. Thereafter, the excess sample was blotted away using blotting paper (Whatman 3MM). The samples were negatively stained with 1% phosphotungstic acid for 10 s, and the excess stain was blotted away as mentioned before. The samples were airdried overnight and imaged using the JEOL JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan) equipped with a LaB_6 filament.

2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism6 software. Significant differences between groups were determined using a t-test for each time point, adjusted for multiple comparisons.

3 Results

3.1 Rosin-functionalized plastic reduces ASFV infectivity

To establish whether rosin-functionalized plastic had an antiviral effect against ASFV, ASFV was incubated with the rosin-functionalized plastic or LDPE plastic for different lengths of time (5, 10, 30, 60, 120, 240, and 480 min). The resulting virus was washed with complete RPMI, and the viral titers were determined using qPCR and HAD_{50} assay.

For all the time points, the number of genome copies detected by qPCR in the flushed-out medium remained constant across the different time points for both plastics, indicating that the virus does not strongly adhere to either of the plastics (Figure 1A). In contrast, the HAD_{50} assay, which determines the titer of viable virus, showed that the viral titer was reduced by 1.3 $\log_{10}$ after 60 min of incubation with the rosin-functionalized plastic. Strikingly, after 120-, 240-, and 480-min incubation, no red blood cell rosettes were detected using the HAD_{50} assay for the virus incubated with the rosin-functionalized plastic, confirming a ~6 $\log_{10}$ reduction for these time points (Figure 1B). In contrast, the viable virus titer of the virus in contact with LDPE plastic was reduced by <1 $\log_{10}$ after 120 min after incubation. The viability of the PAMs used for the HAD_{50} assay was not affected by the flushed medium from either plastic after 4 h of incubation (data not shown). These results suggest that the rosin-functionalized plastic was very efficient in reducing the infectivity of ASFV on its surface.

3.2 Rosin-functionalized plastic affects ASFV morphology

To investigate the effect of incubation with rosin-functionalized plastic on the virus structure, TEM was performed. ASFV was added to the plastic surfaces for 2 h at 37°C. Thereafter, a fixative solution was added directly to the surface for an extended period of time. As the samples were then shipped from the Pirbright Institute to the University of Jyväskylä, Finland, it was likely that many of the viruses had detached from the surface to the fixative solution. Furthermore, we found previously that ASFV was easily detached from the plastic surfaces (Figure 1A). Therefore, the imaging of the viruses was carried out using the fixative solution collected from the plastic samples. After gently sedimenting the viruses using an Airfuge for 30 min, the samples were applied onto TEM grids and negatively stained. Observations under an electron microscope demonstrated that only intact-looking virions (10) were found in the solution from the LPDE plastic samples (Figure 2A). In contrast, we could not find any intact virions from the samples flushed from the rosin-functionalized plastic, suggesting that the majority of the ASFV had undergone significant morphological changes on the rosin-functionalized plastic surface (Figure 2B). These results, together with infectivity measurements, demonstrate efficient antiviral action of the rosin-functionalized plastic within a 2-h time frame.

4 Discussion

The resin acid family covers a spectrum of antimicrobial activities against several micro-organisms, including Gram-positive bacteria, enveloped viruses and fungi (17, 18, 20, 21, 24). Although the mechanism of action is not known, due to the differences in effectivity against Gram-positive bacteria and Gram-negative bacteria, it was hypothesized that differences in the cell membranes are important for the mechanism of action of rosin and resin acids against Gram-positive bacteria (21, 24). Rosin-containing soap also showed virucidal activity against enveloped viruses in 5 min (e.g., influenza A virus, respiratory syncytial virus, and SARS-CoV-2), but failed to affect the non-enveloped encephalomyocarditis virus. The authors thus suggested that the target for the antiviral activity of rosin soap is the viral envelope (17). ASFV is infective *in vitro* with or without the outer envelope and thus it was of interest to investigate whether rosin-functionalized plastic has an effect against this virus.

Indeed, the rosin-functionalized plastic exhibited an antiviral effect against ASFV, starting from 60 min of contact time and achieving complete inactivation after 120 min of contact. Previously, it was shown that the rosin-functionalized plastic efficiently inactivates coronaviruses already after 15 min of contact (19). Even though a

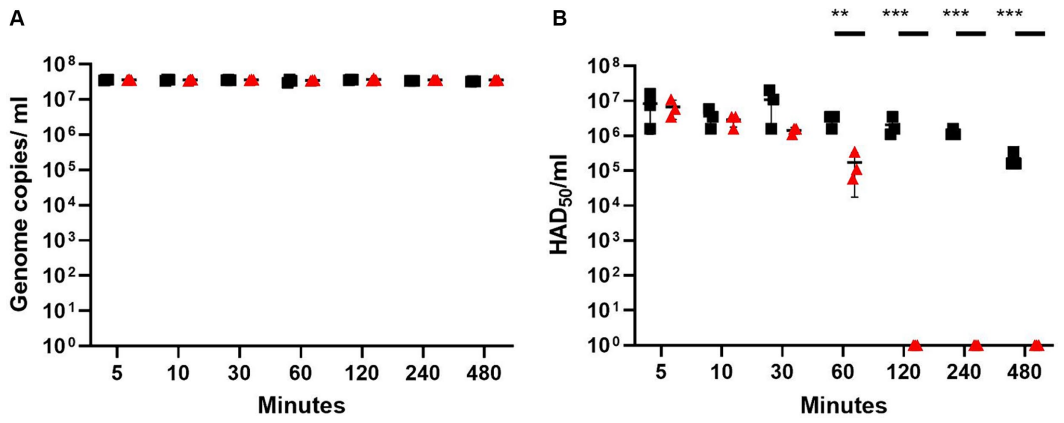

FIGURE 1
ASFV titers after contact with rosin-functionalized (PREXELENT®) or LDPE plastic as determined using qPCR **(A)** or HAD_{50} assay **(B)**. ASFV at $x10^6$ HAD_{50} was incubated with rosin-functionalized plastic (PREXELENT®) (red triangles) or LDPE plastic (black squares) for 5, 10, 30, 60, 120, 240, or 480 min and flushed with RPMI. ASFV genome copies from the flush were determined by qPCR **(A)** and viable virus titers by HAD_{50} assay **(B)**. Statistical significance was determined using t-tests for each time point, adjusted for multiple tests (**$p < 0.01$, *** $p < 0.001$).

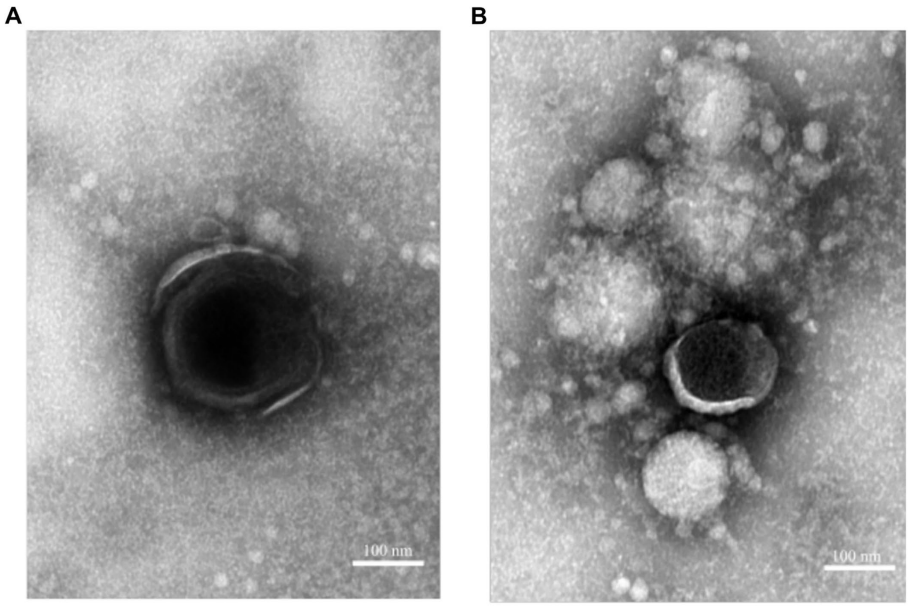

FIGURE 2
ASFV as visualized by TEM incubated with rosin-functionalized plastic (PREXELENT®) **(A)** or LDPE plastic **(B)**. Viruses were gently pelleted down from fixative solution and subjected to negative staining (see Material and Methods section). Bar, 100 nm.

much longer contact time is required to inactivate ASFV, the antiviral activity against ASFV in this study demonstrates that the rosin-functionalized plastic also acts against more complex viruses.

Schroff et al. showed that contact with rosin-functionalized plastic did not lead to apparent structural changes in SARS-COV-2 and seasonal human coronavirus. Although the exact mechanism of action was not elucidated, the viruses were able to bind the host cell surface and enter the endosomes. However, the block in infectivity occurred in the endosomal membrane fusion step between the viral envelope and the endosomal membranes (19). In contrast, TEM imaging performed in this study showed changes in the morphology of ASFV after 2 h of contact with rosin-functionalized plastic. Even though we did not investigate the ability of the virus to bind and enter the host cell, we hypothesize rosin might have different modes of action against enveloped viruses. It would be interesting to investigate the mechanism of action against ASFV in future studies. It would also be interesting to know whether the rosin-functionalized plastic has antiviral activity against other livestock pathogenic viruses.

Rosin-functionalized plastics have promising applications in the livestock sector, given any object or material in contact with infected animals could act as a fomite for virus transmission. The tested functionalized plastic belongs to the low-cost commodity plastics (LDPE) and shows great durability typical of these plastics. As the

plastic May contain a high amount of effective rosin (e.g., 10%), it May remain active for months to years. With the demonstration of antiviral activity against ASFV, a very stable virus, the rosin-functionalized plastic is likely to exhibit antiviral effects against a broad spectrum of livestock pathogenic viruses. Consequently, incorporating rosin-functionalized plastic to diminish viable pathogen loads in fomites could contribute to the reduction of pathogen transmission within and between farms. Carlson et al. (13) demonstrated that the virus survival was largely dependent on soil structure and pH; sandy soil was more optimal for viral stability compared to acidic forest soil. Therefore, it would need to be validated whether the rosin-functionalized plastic is still effective when organic materials are present and/or when cleaning agents and disinfectants are used. This could limit the applicability of the rosin-functionalized plastics to areas or items with minimal organic contamination. The use of effective antiviral surfaces could play a pivotal role in integrated control programs aimed at mitigating infectious diseases. Examples of potential applications on the farm could include door handles, workbenches, tools, and toolboxes used by farm workers and visitors. The plastic May also be applicable in laboratories handling samples potentially containing viruses.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

JH: Conceptualization, Data curation, Writing – original draft, Writing – review & editing, Investigation, Methodology, Supervision. SS: Investigation, Writing – review & editing, Methodology, Writing – original draft. NC: Investigation, Writing – review & editing, Formal analysis, Methodology. MH: Investigation, Writing – review & editing, Methodology. LD: Methodology, Writing – review & editing, Conceptualization, Investigation. VM: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing, Methodology.

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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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Stability of a surrogate African swine fever-like algal virus in corn- and soybean-based feed ingredients during extended storage and *in vitro* digestion processes

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Prevention of transmission of African swine fever virus (ASFV) through contaminated feed ingredients and complete feed is an important component of biosecurity protocols for global feed supply chains. Use of extended storage times for feed ingredients has become a popular and emerging mitigation strategy that may allow partial inactivation of ASFV before manufacturing swine feeds. However, the effectiveness of this strategy is unclear because limited studies have been conducted using diverse methodologies and insufficiently sensitive measures of virus viability of only a few types of feed matrices. Therefore, interpretation of results from these studies has made providing prudent recommendations difficult. Furthermore, although a few studies have shown that feed is a plausible route of transmission of ASFV to pigs, there are conflicting findings on the infectivity of ASFV that may be present in feed, which may be related to the extent that ASFV is degraded in the pig's digestive system after it is consumed. Therefore, the objectives of this study were to use a surrogate ASFV-like algal virus (*Emiliania huxleyi*; EhV) to determine stability in corn- and soybean-based feed ingredients and complete feed during a 120-day storage period at temperatures up to 34°C, and EhV survival in various feed matrices during three stages of an *in vitro* digestion process. Results indicated that inoculating corn- and soybean-based feed ingredients and complete feed with EhV and storing them at 4°C, 24°C, or 34°C for up to 120 days did not result in the complete inactivation of EhV in any of these matrices. Because EhV has similar environmental and thermal resilience to ASFV, these results indicate that both viruses can maintain viability in various feed matrices during long-term storage and suggest that extending storage time up to 120 days is not an effective mitigation practice against ASFV. We also determined that between approximately 5- to more than 7-log (99.999 to 99.99999%) reductions in EhV in various feed matrices occur during the entire *in vitro* digestion and fermentation process. These reductions appear to be correlated with the chemical composition of the matrices, potentially explaining inconsistencies in ASFV infection when pigs consume infectious doses of contaminated feed.

KEYWORDS

African swine fever virus, corn-based ingredients, *Emiliania huxleyi* virus, extended storage, *in vitro* digestibility, soybean-based ingredients, viability PCR

1 Introduction

African swine fever virus (ASFV) continues to infect hundreds of thousands of pigs in numerous countries worldwide, causing enormous economic losses and significantly increasing the environmental footprint of pork production systems (1). Although some progress has been made in vaccine development, it has yet to become a viable disease prevention and control strategy (2–5). Furthermore, there are no treatments to control ASFV, but some antiviral feed additives have been shown to be effective for partially inactivating ASFV in various feed ingredients and complete feeds under experimental conditions (6, 7). As a result, the most prudent course of action to prevent the spread and subsequent infection is through strict biosecurity protocols (8).

Although the likelihood of transmission and subsequent ASFV infection through feed ingredients and complete feeds is low relative to direct exposure to infected pigs, carcasses, tissues, and body fluids (9, 10), it remains a plausible route that has generated considerable research during the past few years (11–13). Unfortunately, there is no standardized analysis or monitoring system to determine the potential presence, concentration, and stability of ASFV in contaminated ingredients (14). As a result, the use of extended storage times has become a popular and emerging approach to partially inactivate ASFV and other swine viruses that may be present in feed ingredients (14). However, the methodologies used to determine the effectiveness of storage time and temperature have been limited to only a few ingredients (i.e., soybean meal) and have led to highly variable results that are difficult to interpret (13, 14). Lack of sensitivity to detect viable ASFV has contributed to different interpretation of results. Viable ASFV is defined as structurally intact virus particles that can still be infectious when taken up via the macropinocytotic infection route (15). Furthermore, ASFV is much more thermally resilient than previous studies (16) have shown when viability PCR is used (15). We have developed a surrogate assay using an ASFV-like algal virus (*Emiliania huxleyi*; EhV) to simulate ASFV in feed matrices (15, 17). We have also developed a modified *in vitro* digestibility procedure to evaluate the digestion and fermentation of various types of feed ingredients in pigs (18).

Compared with other feed ingredients, soybean meal appears to have unique properties that enable ASFV survival (19, 20) under simulated conditions of a 30-day transoceanic transport and enables ASFV to survive for many months of storage at temperatures up to 35°C (21). Similarly, Palowski et al. (17) showed that, when using EhV as a surrogate for ASFV, no degradation was detected in conventional and organic soybean meal and complete feed samples after a 23-day truck transportation event. Furthermore, Palowski et al. (17) also showed that the majority of EhV remains bound to soybean meal after extraction for PCR or bioassay analysis, which makes soybean meal an ingredient of potential concern for transmission of ASFV. In addition, although corn is the predominant ingredient used in swine diets around the world, it has not been evaluated in ASFV storage studies to the same extent as soybean meal, nor have other types of corn co-products used in swine diets been evaluated.

Moreover, there are conflicting findings on the infectivity of ASFV post-extraction from feed, which may be related to the extent that ASFV is degraded in the pig's digestive system after it is consumed. Two studies have shown that feed and water can be routes of ASFV transmission. Niederwerder et al. (22) determined the minimum infectious dose of ASFV in feed to be 10^4 $TCID_{50}$ with a minimum dose of 10^0 $TCID_{50}$ for liquid. However, Blázquez et al. (23) reported that the minimum infectious dose of ASFV is greater than 10^5 because feeding diets inoculated with 10^5 $TCID_{50}$ of ASFV in liquid plasma for 14 consecutive days failed to cause disease. Reasons for these conflicting results are unclear but may be related to different feed constituents used in each study or due to virus survival during the various stages of the digestive process. No studies have attempted to determine the fate of ASFV-contaminated feed ingredients in the pig gastrointestinal tract during the digestion and fermentation process. Therefore, the objectives of this study were to (1) evaluate survival of EhV, as a surrogate for ASFV, in corn- and soybean-based ingredients and complete feed at different storage temperatures up to 34°C during a 120-day storage period, and (2) determine the survival of EhV in corn- and soybean-based ingredients and complete feed during the simulated *in vitro* hydrolysis and fermentation stages of the digestive process in pigs.

2 Materials and methods

2.1 Sample collection

Representative samples of dehulled, solvent-extracted soybean meal, soybean hulls, extruded soybean meal, corn grain, corn distillers dried grains with solubles, high protein distillers dried grains, and corn fermented protein were obtained from commercial industry sources. In addition, a complete diet consisting of corn (44.9%), solvent-extracted soybean meal (22.9%), corn distillers dried grains with solubles (29.7%), and minerals and vitamins (2.5%) was manufactured to simulate a typical commercial swine grower diet (24).

2.2 Chemical analysis of ingredients and complete feed

The chemical composition (i.e., moisture, crude protein, ether extract, neutral detergent fiber, and ash content) and water activity in each of the seven ingredients and complete feed were determined on day 0 for use in subsequent correlation analysis to explore potential associations between chemical composition of ingredients and virus inactivation rate. All ingredients and complete feed were subsampled, and samples were submitted to the University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, United States) for chemical analyses. Samples were analyzed using AOAC (25) procedures for crude protein (CP; Method 984.13), ether extract (EE; Method 920.39), ash (Method 942.05), and neutral detergent fiber (26). Dry matter content and water activity of samples were measured at the University of Minnesota. Water activity was assessed using a Decagon Pawkit (METER Group, Pullman, WA), and dry matter content was determined following the NFTA 2.2.2.5 method (27).

2.3 Surrogate virus assay and EhV stock

Access to ASFV is highly restricted and requires adhering to strict biosecurity protocols in government-approved high biosecurity

research facilities (BSL-3). Therefore, we developed a surrogate virus assay (RISNA) using EhV to safely and accurately evaluate ASFV survival and mitigation in feed ingredients. The RISNA assay was used to assess EhV inactivation in feed ingredient and complete feed matrices for the storage stability and *in vitro* digestibility experiments. Previous studies have shown remarkable structural (28–30) and functional (15) similarities between ASFV and EhV, which makes EhV a suitable, safe surrogate for these types of experiments.

The EhV strain used in the current study (EhV-86) was provided by Dr. Martinez-Martinez laboratory (Bigelow – Laboratory for Ocean Sciences, East Boothbay, Maine). It was cultured in Alga-Gro® Seawater Medium (Carolina Biological Supply Company, Burlington, North Carolina) in a 15°C incubator until lysis occurred, which was observed after 4 days. The lysate was then filtered through a 0.45 µm filter (Nalgene™ Rapid-Flow™ Bottle Top Filters, ThermoFisher Scientific, MA, US) to remove cell debris. The filtered lysate was aliquoted, titered using flow cytometry, and stored in the dark at 4°C until use.Virus Viability Assay, DNA extraction and qPCR assay.

Platinum IV chloride (Pt_4CL) was chosen as a suitable alternative reagent to replace PMAxx for assessing viable virus particles (31), and a pilot study was conducted to determine the optimal concentration of Pt_4CL to ensure accurate estimation of the EhV viability. Results from this study showed that a dose of 1 mM Pt_4CL provided a similar estimation of EhV viability as that achieved using 100 µM PMAxx (Supplementary Figure 1). Consequently, the viability assay was performed as described by Balestreri et al. (15), replacing PMAxx with 1 mM Pt4CL. Since PMAxx is a dye that requires light activation, the step of exposing samples to light for 30 min to cross-link PMAxx to DNA or RNA was also removed from the protocol. All other aspects of the assay procedure remained unchanged.

Viral DNA was isolated using automated extraction with the NucleoMag Virus kit (Macherey-Nagel, Düren, Germany) and a Magnetic Particle Processor (KingFisher Flex, Thermo Fisher Scientific, United States), following the manufacturer's instructions. A sample volume of 200 µL with a 1 mM Pt4CL concentration was used, with an elution volume of 50 µL in molecular-grade water. Quantitative PCR was conducted using QuantiNova SYBR Green PCR kit (Qiagen, CA, United States) using the following conditions: 2 min at 95°C followed by 40 cycles of 5 s at 95°C and 10 s at 60°C (reaction mix components: SYBR Green PCR Master Mix, primer pair, molecular grade water, and 1 µL DNA template). The PCR assay was conducted using a QuantStudio 3 Real-Time PCR (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, United States). Standards for the EhV qPCR assays were created as described by Balestreri et al. (15).

2.4 Experiment I—assessment of EhV viability under storage conditions

Samples of all feed ingredients and complete feed were inoculated with EhV on day 0 and stored at three different temperatures (4, 24 and 34°C) for up to 120 days to determine inactivation kinetics of EhV under simulated storage conditions. The storage temperatures were chosen based on historical temperature and relative humidity data for both land and oceanic segments of two 37-day transboundary shipping models for transporting feed ingredients from China and Europe to the United States (19). The range of temperatures for the China-Pacific-United States route was between 4 to 10°C (December – January conditions), and the range of temperatures for the Europe-Atlantic-USA was between 4 to 20°C (April–May conditions). In addition, a maximum temperature of 34°C was used to represent summer storage conditions in enclosed silos in the United States.

One set of triplicate samples were prepared by adding 1 g of each feed matrix to sterile 15 mL Falcon tubes (CorningTM Falcon 15 mL Conical Centrifuge Tubes, ThermoFisher Scientific, MA) and served as the positive controls (matrix or blank + EhV), while another set of triplicate samples of 1 g of each matrix served as negative controls (matrix or blank + medium). For the positive control samples, 200 µL of EhV-86 filtrate (1×10^8 cells/mL) was added to each tube containing 1 g of each matrix, and 200 µL of AlgaGro® Seawater Medium (Carolina Biological Supplement Company, North Carolina, United States) was added to each 1 g matrix in tubes for the negative control samples. AlgaGro® Seawater Medium was used as a negative control because it is the medium in which the EhV virus grows and serves as the base solution for the positive control. In addition, 1×10^8 cells/mL of stock virus was added to empty 15 mL tubes as the baseline for assessing the survival of EhV without the effect of the matrix under the simulated storage conditions. Both sets of triplicate samples of each feed matrix were placed in environmental chambers for each temperature (4, 24 and 34°C) and timepoint (1, 5, 60 and 120 days). Thus, a total of 648 samples were used in the experiment [9 treatments (8 matrices and a blank) × 6 samples per treatment (3 positive +3 negative controls) × 3 temperatures (4, 24 and 34°C) × 4 time points (1, 5, 60 and 120 days)]. At each time point, the two sets of triplicate treatments (positive and negative controls) for each temperature were removed from the environmental chambers to determine viable EhV concentrations.

2.5 Experiment II—assessment of EhV viability after *in vitro* stomach and small intestine digestion

An *in vitro* digestion assay (18) was modified to accurately estimate EhV viability on an experimental scale by determining the appropriate combination of virus inoculum concentration, matrix weight, and buffer volumes to ensure precise PCR measurement without causing dilution effects. This modified protocol was used to determine the fate of EhV during *in vitro* digestion of inoculated ingredients and complete feed after two enzymatic hydrolysis steps simulating stomach digestion (pepsin hydrolysis) and small intestine digestion (pancreatin hydrolysis).

Two sets of triplicate samples were prepared, which consisted of positive controls (matrix or blank + EhV) and negative controls (matrix or blank + medium). Additionally, the viability of EhV during the digestive process in the absence of a feed matrix was assessed, resulting in a total of nine treatments (eight matrices and one blank). All feed matrices were ground to pass a 1 mm mesh screen before undergoing *in vitro* pepsin and pancreatin hydrolysis. Approximately 100 ± 5 mg of each sample was weighed into sterile 15 mL tubes (Corning™ Falcon 15 mL Conical Centrifuge Tubes, ThermoFisher Scientific, MA). For the positive control samples, 2 mL of EhV-86 filtrate (1×10^8 cells/mL) was added to each tube, and for the negative control samples, 2 mL of AlgaGro® Seawater Medium (Carolina Biological Supplement Company, North Carolina, United States) was

added to each tube. Next, 6 mL of a previously prepared pepsin solution, as described by Huang et al. (18), was immediately added to each tube, and the tubes were placed in a water bath at 39 ± 0.5°C for 2 h under gentle agitation. At the end of the 2 h incubation period, samples were centrifuged at 4,700 rpm for 10 min. The concentration of viable EhV was then measured and calculated as previously described by Balestreri et al. (15). Immediately after the 2 h incubation period, the same samples were used to determine the effect of pancreatic hydrolysis by adding 2.5 mL of a previously prepared pancreatin solution (18) to each tube, placing them in a water bath at 39 ± 0.5°C for 4 h under gentle agitation, and determining the concentration of viable EhV as previously described by Balestreri et al. (15).

2.6 Experiment III—assessment of EhV viability after *in vitro* large intestine fermentation

Residues from enzymatic hydrolysis of each feed matrix without EhV inoculation were used to determine EhV viability during *in vitro* large intestine fermentation using procedures described by Huang et al. (18). Briefly, about 100 ± 5 mg of each hydrolyzed residue was weighed in a 125 mL serum bottle with rubber stoppers which contained 10 mL buffer solution with 5% fecal inoculum. The protocol for preparing the fecal inoculum was previously described by Huang et al. (18). The viability of EhV during the fermentation process was assessed in the absence of a feed matrix, in the fecal inoculum (fermentation buffer solution treatment), and in the EhV inoculated feed matrix residues, for a total of 10 treatments (eight feed matrices, fermentation buffer, and fecal inoculum). For each treatment, two sets of triplicates were used that consisted of a set of positive controls (matrix or solutions + EhV) and a set of negative controls (matrix or solutions + seawater medium). Immediately after adding the EhV and the fecal inoculum or the buffer solution, the bottles were placed in a water bath at 39 ± 0.5°C for 24 h. At the end of the 24 h incubation period, 200 µL samples were collected from each tube for the assessment of EhV viability as previously described in Balestreri et al. (15).

2.7 Statistical analysis

A simple linear regression analysis was used to analyze EhV viability data from Experiment I. In Experiment II, an ANOVA was used to assess differences in EhV viability during stomach and small intestine digestion among various feed matrices, and an independent two-sample t-test was used to determine if there were significant differences in EhV viability between the stomach-only and the stomach + small intestine digestion processes. Similarly, data analysis from Experiment III involved an ANOVA to evaluate differences in EhV viability during large intestine fermentation. A post-hoc Tukey's test was used to assess differences between treatments in Experiments II and III. Experimental error associated with the viability PCR method was estimated to be in the ±1 log reduction range, which was determined empirically by analyzing multiple replicates of samples with known viral concentrations. Given that a single data point is derived from a PCR amplification plot (i.e., known as the threshold cycles reported as CT or Cq values), and that the error in CT over an exponential phase of amplification (where CT values are taken) is equivalent to doubling events, a 3-point difference in a CT value is approximately equivalent to a 10-fold change in the quantity of viral genetic material. This means that a differences in a single CT value between biological replicates can have an error greater than 30% entirely due to cycling inefficiencies, which can occur from pipetting errors, properties of the polymerase, or the characteristics of a given matrix from where the virus was extracted. Responses falling within this threshold were considered negligible and treated as zero for data analysis purposes, irrespective of the statistical methodology employed. All statistical tests were conducted at a significance level of $p < 0.05$. Data visualization and statistical analyses were conducted using RStudio (version 2024.04.1) and R (version 4.2.2). The R package of dplyr (version 1.1.2) was used for data manipulation, ggplot2 (version 3.5.1) was used for data visualization, and emmeans (version 1.8.6) and multcomp (version 1.4.25) were used for contrasts and multiple comparisons.

3 Results

3.1 Experiment I—assessment of EhV viability under storage conditions

Viability of EhV was determined in all corn- and soybean-based ingredient and complete feed samples stored at 4°C, 24°C, and 34°C on days 1, 5, 60, and 120 post-inoculation (Figure 1). Statistically significant linear reductions were observed for viable EhV concentrations across time and temperature conditions evaluated in this study. However, despite the statistical significance of these responses, the differences observed did not exceed the margin of error for the PCR viability assay, which was estimated to be ±1 log concentration across these time and temperature conditions (Table 1).

Figure 2A presents the data on viable EhV across a 120-day storage period. Although a statistically significant trend was observed in viable EhV across all matrices combined ($p = 0.006$), the reduction in viable EhV concentration was 0.2 log, which falls within the margin of error for the PCR viability assay, and therefore the linear relationship is not depicted in the plot. Figure 2B illustrates the relationship between EhV viability and temperature, showing no significant differences in EhV inactivation across temperatures ($p = 0.483$) for all matrices combined. Thus, no reductions in EhV viability exceeding a 1 log concentration were detected at any temperature or for any matrix throughout the 120-day experimental period.

The chemical composition and water activity of the feed matrices evaluated in this study were determined (Table 2). There were wide ranges in CP (7.8–49.3%), EE (0.8–7.27%), ash (1.28–5.69%), NDF (8.7–58.6%), and water activity (0.32–0.70 a_w). Ash concentration of the corn- and soybean-based feed ingredients evaluated was linearly associated ($p = 0.034$) with average EhV concentration during all storage time points and temperatures combined (Figure 3). However, the predicted potential protective effect of ash content in feed ingredients on EhV did not exceed the calculated experimental error of ±1 log. No other significant correlations were observed regarding the chemical composition of the feed matrices and viable EhV concentrations. Because no EhV inactivation was observed under any of the tested storage conditions (4°C, 24°C, and 34°C at 1, 5, 60, and 120 days of storage), no correlations could be calculated between chemical composition, water activity, and EhV inactivation.

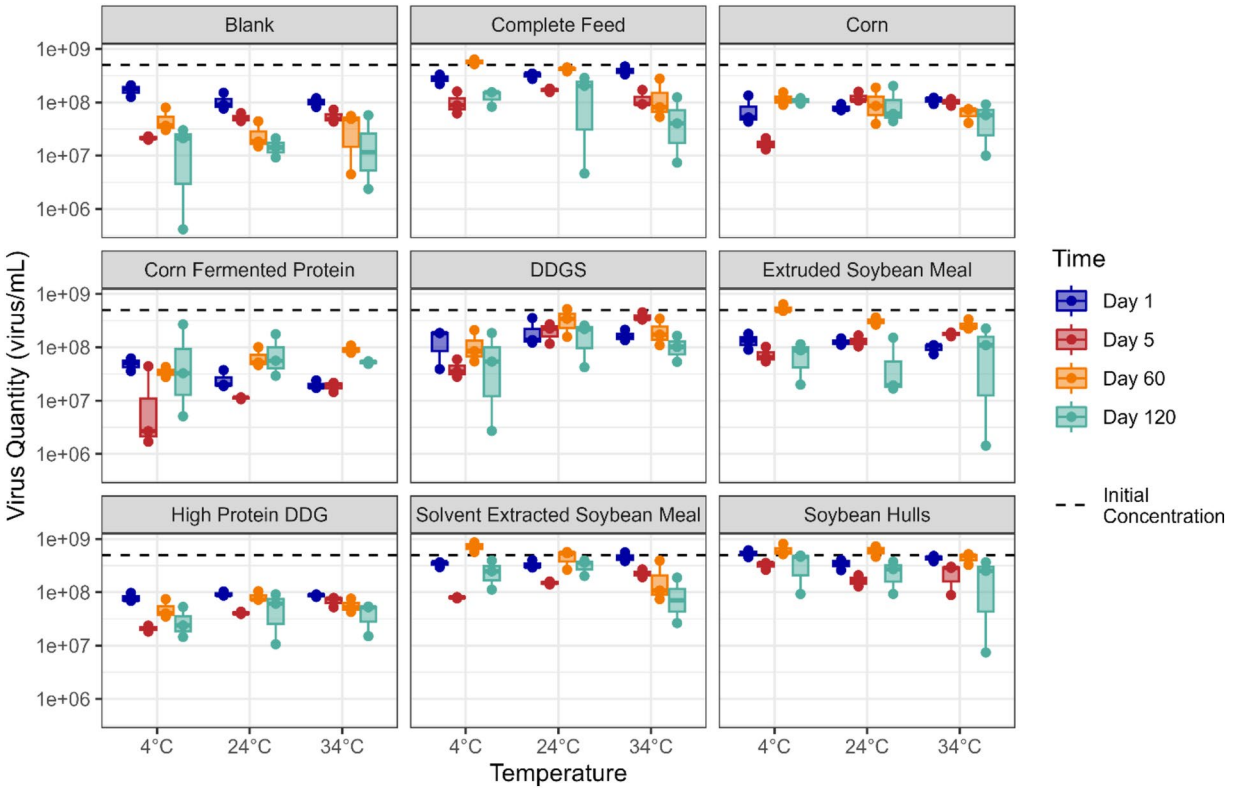

FIGURE 1
Boxplot illustrating the quantity (virus/mL) of viable EhV in different feed matrixes at different temperatures (x-axis) and timepoints (colors indicate days post-inoculation). The red dashed line denotes the EhV virus quantity at day 0.

TABLE 1 Effect of time and temperature on viable EhV concentration difference.

Feed Matrix	Effect of temperature (Δ Temperature[1]) at				Effect of time (Δ Time[2]) at		
	Day 1	Day 5	Day 60	Day 120	4°C	24°C	34°C
Logarithmic concentration difference							
Blank	−0.24*	0.42*	−0.31	0.32	−0.95	−0.75*	−0.86*
Complete Feed	0.14*	0.12	−0.66*	−0.56	−0.02	−0.48	−0.81*
Corn	0.20	0.86*	−0.26	−0.41	0.55*	−0.08	−0.46*
Corn Fermented Protein	−0.40*	0.49	0.42*	0.20	0.33	0.66*	0.53*
DDGS	0.19	0.99*	0.34	0.57	−0.30	−0.10	−0.39*
Extruded Soybean Meal	−0.11	0.39*	−0.32*	−0.26	−0.08	−0.44	−0.50
High Protein DDG	0.05	0.50*	0.11	0.14	−0.18	−0.17	−0.35*
Solvent Extracted Soybean Meal	0.10	0.45*	−0.64*	−0.32	0.21	0.17	−0.68*
Soybean Hulls	−0.12	−0.24	−0.13	−0.45	−0.14	0.02	−0.47

[1]Viable EhV concentration at 4°C minus viable EhV concentration at 34°C; calculated using linear regression.
[2]Viable EhV concentration at d 1 minus viable EhV concentration at d 120; calculated using linear regression.
*Linear effect ($p < 0.05$).

3.2 Experiment II—assessment of EhV viability after *in vitro* stomach and small intestine digestion

Pepsin + pancreatin digestion (simulating stomach and small intestine conditions) resulted in an average reduction of 2.8 log units in EhV viability among all feed matrices, but soybean-based ingredients had a greater protective effect on virus viability than corn and corn co-products (Figure 4). However, when comparing the virus stability in stomach-only (pepsin hydrolysis) with combined stomach + small intestine (pepsin + pancreatin hydrolysis) conditions, no differences exceeding the estimated

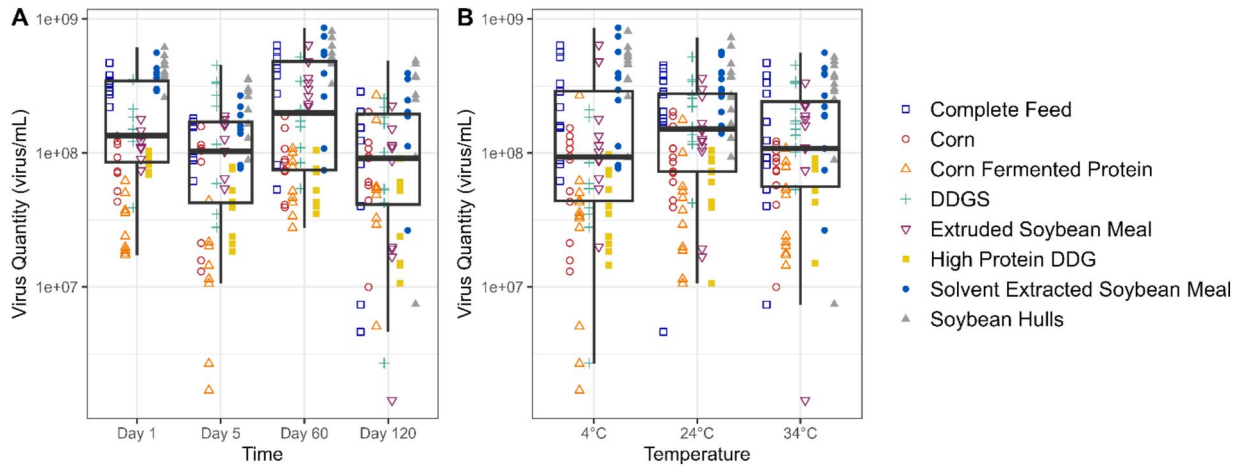

FIGURE 2
Boxplot depicting the quantity (virus/mL) of viable EhV in different feed matrixes at different storage time points **(A)** and temperatures **(B)**.

TABLE 2 **Nutritional composition of feed ingredients and complete feed.**

Feed Matrix	Nutritional component, %					Water activity a_w
	Dry matter	Crude protein	Ether extract	Ash	Neutral detergent fiber	
Complete Feed	87.4	21.6	2.47	5.06	17.8	0.70
Corn	88.5	7.83	3.04	1.28	12.8	0.59
Corn Fermented Protein	93.1	49.2	3.55	3.37	42.7	0.53
DDGS	87.6	30.7	6.77	4.41	30.3	0.62
Extruded Soybean Meal	95.6	44.7	7.27	5.68	11.4	0.32
High Protein DDG	93.4	49.3	6.99	2.14	36.6	0.51
Solvent Extracted Soybean Meal	87.8	47.2	1.17	5.47	8.66	0.67
Soybean Hulls	90.4	9.89	0.8	4.63	58.6	0.56

experimental error of the viability PCR method (±1 log) were observed.

3.3 Experiment III—assessment of EhV viability after *in vitro* large intestine fermentation

An average reduction of 2.68 log units in viable EhV concentrations was observed in the buffer solution with no feed matrix or fecal inoculum (Figure 5). Additionally, fermentation with buffer and fecal inoculum but without predigested substrate resulted in an average reduction of 3.8 log units in viable EhV concentration over a 24 h time period. The average viable EhV concentration in solvent extracted soybean meal was reduced by 3.53 log units during *in vitro* fermentation, while all other feed matrices had an average reduction of 4.1 log units during the 24 h fermentation period. However, it should be noted that these calculated reductions are minimum values, and the actual virus inactivation may have been greater. Exact reductions could not be determined due to the lower limit of detection (LOD) in the experiment. No statistically significant differences were observed among treatments in EhV viability during the *in vitro* fermentation process ($p = 0.097$).

4 Discussion

No system for monitoring or standardized testing of the potential presence and concentration of ASFV in feed ingredients exists in global or domestic feed supply chains. As a result, very little is known about the likelihood of ASFV transmission in feed. In fact, a recent risk assessment on ASFV transmission conducted by Bergmann et al. (32) did not include feed as a potential factor. Among the limited number of other risk assessments conducted for feed ingredients, two have been qualitative without indicating that there is a high degree of uncertainty (33, 34), one was quantitative but only included imported corn and soybean meal from ASFV-positive countries into the United States (12), and one was quantitative but focused only on disease status of the country of origin (11). Because of the high uncertainty of knowing whether a feed ingredient imported from an ASFV-positive country is contaminated, and the need for high biosecurity due to the lack of commercially available preventive

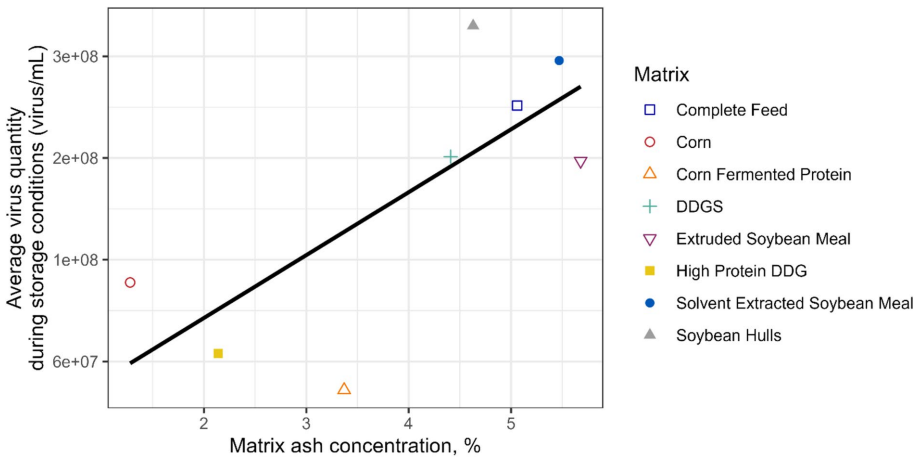

FIGURE 3
A linear association (p = 0.034) was observed between the feed matrix ash concentration and the average viable EhV quantity during the storage conditions considering all time points and temperatures combined.

vaccines, use of extended storage times has become a popular mitigation approach for partial ASFV inactivation if it is present. Overall, current evidence of ASFV transmission through feed under field conditions is circumstantial. Confronted with no monitoring systems, food safety professionals may assume potential virus contamination and consequently use a microbiological risk assessment model as a prudent approach to address the issue (35). A microbiological risk assessment can be used to calculate scenarios for initial virus concentration and the appropriate amount of virus inactivation for the performance objective. An example of using a performance objective to assess risk of ASFV in spray dried porcine plasma was recently published (36). However, very few studies report the d-values of virus inactivation and likelihood of contamination in the scientific literature.

4.1 Experiment I—assessment of EhV viability under storage conditions

Although statistically significant linear reductions were observed for viable EhV concentrations across time and temperature conditions evaluated in this study, the differences observed did not exceed the margin of error for the PCR viability assay, which was estimated to be ±1 log concentration across these time and temperature conditions. As a result, it is uncertain whether the differences observed represent actual virus inactivation or if they are artifacts of experimental error. Although an initial reduction of 0.5 log in viable EhV concentration occurred between day 1 and day 5, when considering the viable EhV concentrations at day 60 and day 120, this reduction appears to be related to experimental error rather than virus inactivation.

No reductions in EhV viability exceeding a 1 log concentration were detected for any matrix at any temperature throughout the 120-day experimental period because the experimental error was within the range of 1 log concentration. Therefore, these results indicate that storing corn- and soybean-based feed ingredients and completed feed inoculated with EhV at 4°C, 24°C, or 34°C for up to 120 days had a negligible effect on inactivation of EhV in all feed ingredients and complete feed matrices evaluated. Because EhV is an ASFV-like virus, these results indicate that ASFV can maintain viability in various feed matrices during long-term storage and suggest that extending storage time alone may not be an effective mitigation practice for ASFV.

Only a few studies have been conducted to determine the effect of storage time and temperature on ASFV survival in feed ingredients and complete feed. Unfortunately, the effectiveness of extended storage is difficult to interpret because of the analytical methods used to determine ASFV concentration. Stoian et al. (20) reported half-life values for conventional and organic soybean meal, complete feed, pet foods, choline, and pork sausage casings that were experimentally inoculated with 10^5 $TCID_{50}$, which ranged from 9.6 (conventional soybean meal) to 14.2 (complete feed) days during a simulated 30-day transoceanic shipment at an average temperature of 12.3°C and average relative humidity of 74.1%. Half-life is an estimate of the amount of time it takes for half of the virus to be inactivated but does not indicate viability or infectivity of the virus. Fischer et al. (37) evaluated the effects of inoculating spray-dried porcine plasma with 10^6 HAD_{50}/mL and storing it for up to 35 days at 4°C and 21°C. For this feed matrix, the ASFV concentration was reduced by >5.7 log after 2 weeks of storage at 21°C. Although the HAD_{50} assay is used as a method for estimating the infectivity of ASFV, pigs can become infected after exposure to only a few virus particles while others may require a concentration of 10^7 HAD_{50} for infection. Furthermore, the HAD_{50} assay only measures the viruses that can attach to red blood cells, but viruses that lose this HA phenotype are also infectious. Therefore, the HAD_{50} method is not a definitive measure of ASFV infectivity. For example, Niederwerder et al. (22) reported that although a low dose of 10^2 HAD_{50} did not cause ASFV infection, a moderate (10^4) dose was sufficient to cause infection. In another study, Niederwerder et al. (21) determined the stability of an ASFV Georgia 2007 isolate in complete feed, soybean meal, and ground corn cobs when exposed to 4°C, 20°C, and 35°C for up to 365 days using qPCR, virus isolation, and swine bioassays. Soybean meal required the longest amount of time for reduction in ASFV infectivity followed by complete feed and corn cob particles, which led to the recommendation that ASFV-contaminated feed be stored for >112 days at 4°C, >21 days at 20°C, and > 7 days at 35°C. These

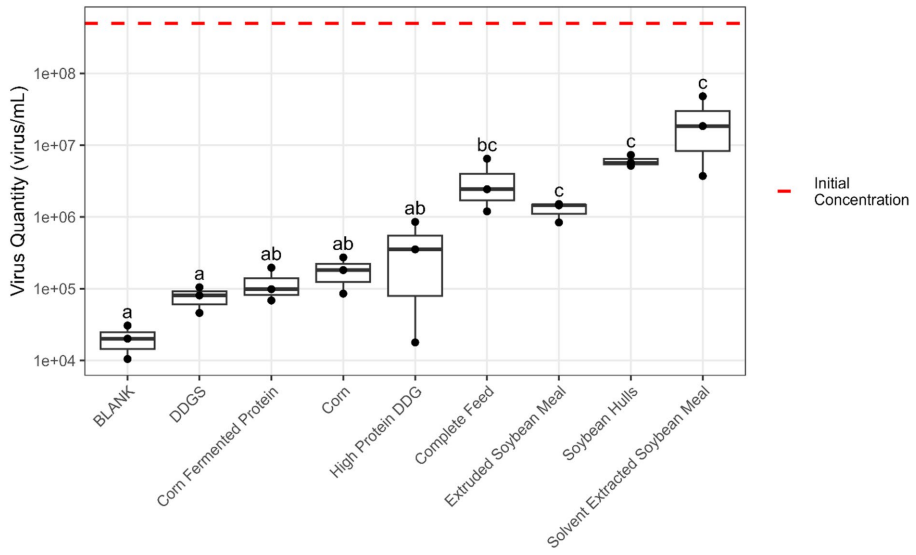

FIGURE 4
Boxplot of the quantity of viable EhV (virus/mL) after the 6 h *in vitro* digestion process using pepsin + pancreatin to simulate stomach and small intestine digestion. The red dashed line represents the initial EhV virus concentration. Matrices without a common letter are significantly different ($p < 0.05$) according to the Tukey test.

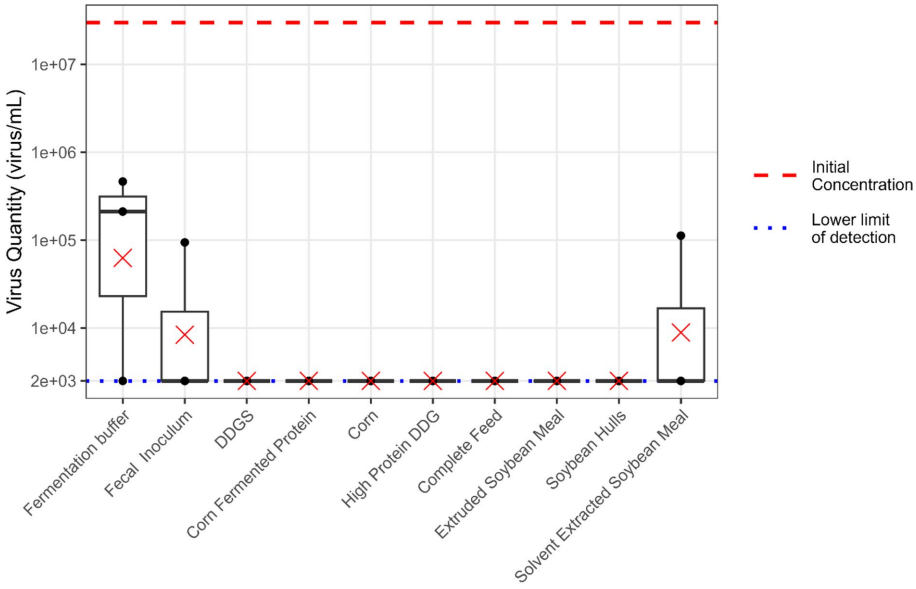

FIGURE 5
Boxplot illustrating the quantity of viable EhV (virus/mL) during 24 h *in vitro* large intestine fermentation. The red dashed line represents the initial EhV virus concentration. The blue dotted line indicates the lower limit of detection for EhV viability. Mean values are denoted by "×" symbols for each feed matrix.

recommendations are not consistent with those observed in the current study.

Two other studies reported conflicting results when various types of feed ingredients were exposed to higher temperatures (>40°C) for shorter periods of time (< 2 h). Fischer et al. (38) determined ASFV concentrations in wheat, barley, rye, triticale, corn, and peas inoculated with ASFV-infected blood (10^6 HAD_{50}/mL) and reported that ASFV was detected in all samples by PCR when dried at 20°C for 2 h and incubated for 1 h at 75°C, but no infectivity, as measured by HAD_{50} and virus isolation, was observed after 2 h of storage at 20°C. Songkasupa et al. (39) used HAD_{50} to quantify ASFV concentrations, calculate D values (time required to reduce ASFV by 1 log at a specific temperature), and develop models to predict ASFV inactivation in corn, soybean meal, and meat and bone meal when exposed to temperatures of 60, 70, 80, and 90°C for 20 min. They observed no differences in D values and heat resistance among

ingredients. Furthermore, exposure of feed ingredients to high temperatures for longer periods, such as the multiple weeks spanned by the current study, results in degradation of lipids, proteins, and vitamins (13). Therefore, holding feed ingredients at high temperatures during long storage periods is not a feasible approach for inactivating ASFV.

Although the ash concentration of the corn- and soybean-based feed ingredients was linearly associated ($p = 0.034$) with average EhV concentration during all storage time points and temperatures combined, the predicted potential protective effect of ash content in feed ingredients on EhV did not exceed the calculated experimental error of ±1 log which suggests that the observed effect may be attributable to experimental error rather than a true protective effect. No other significant responses were observed regarding the chemical composition of the feed matrices and viable EhV concentrations. Physical characteristics and chemical composition of feed matrices are likely to play an important role in EhV and ASFV survival and inactivation, but very little is known about these potential effects. A moderate correlation has been observed between moisture concentration of feed ingredients and increased survival of porcine delta coronavirus ($r = 0.48$) and transmissible gastroenteritis virus (40). Water activity of food matrices is a good predictor of thermal resistance of bacterial pathogens in foods (41), but is rarely determined in feed ingredients. A previous study showed that water activity was greater in soybean meal, barley, rapeseed cake, and corn that was milled to a coarse particle size compared with fine particle size, with coarse milled soybean meal having the greatest water activity (42). Solvent extracted soybean meal (0.67 a_w) and complete feed (0.70 a_w) had the greatest water activity among feed matrices evaluated in the current study. Other compounds such as isoflavones and saponins in soybean meal (43) and copper and zinc (44–49) have been shown to have antiviral and antimicrobial properties. The addition of sodium chloride has been shown to be effective in partially inactivating porcine delta coronavirus (50) and porcine epidemic diarrhea virus (51) in complete feed. However, because no EhV inactivation was observed under any time and temperature conditions evaluated in this study, no correlations between composition, water activity, and EhV inactivation could be estimated. Nonetheless, this phenomenon warrants further investigation because it has the potential to explain unknown dynamics of ASFV infection from feed consumption.

4.2 Experiment II—assessment of EhV viability after *in vitro* stomach and small intestine digestion

Although there is no direct evidence indicating that feeding naturally contaminated feed to pigs causes disease under field conditions, Oļševskis et al. (52) suggested that feeding swill and potentially contaminated fresh grass or crops were probable causes of ASFV outbreaks on some swine farms in Latvia but provided no definitive evidence for this potential route of transmission. Similarly, Wen et al. (53) was unable to isolate live ASFV from dried blood meal samples used in swine feed, but inferred it was a "highly likely" source for the spread of ASFV in China. Zhai et al. (54) also suggested that feed was a cause of ASFV transmission in China despite providing any quantitative evidence. Likewise, Gebhardt et al. (55) collected 54 samples of complete feed and feed ingredients from a feed mill serving multiple internal and external swine production sites that were contaminated with ASFV, but none of the samples tested positive for ASFV using a PCR assay. However, these researchers noted that all feed manufactured for internal use contained a commercial formaldehyde-based feed additive used at the recommended dose. A commercially available formaldehyde product is approved for use in Salmonella control in the United States but not for ASFV. Formaldehyde may inactivate ASFV by inducing DNA damage, cell damage, and interference with virus replication. These effects are dependent on concentration which is best described as the decimal-concentration [d-value; (56)]. It is also important to note that the DNA damage effects of formaldehyde-based products can still render the DNA detectable by PCR (7). Therefore, the negative PCR result observed by Gerhardt et al. may indicate a genuine absence of ASFV in the samples. Unger et al. (57) showed a correlation between the frequency of ASFV-infected pigs and their proximity to bodies of water but provided no direct evidence to indicate ASFV-contaminated water was the cause of infection. However, studies have shown that ASFV can survive and remain infectious in experimentally inoculated feed for up to 365 days (21) and for up to 42 days in river water at 4°C (58).

Despite the lack of direct evidence for transmission of ASFV through feed under field conditions, Niederwerder et al. (22) showed that feed and water can be routes of ASFV transmission by determining the minimum infectious dose of 10^4 $TCID_{50}$ in feed and 10^0 $TCID_{50}$ in liquid. However, Blázquez et al. (23) reported that the minimum infectious dose of ASFV is greater than 10^5 because feeding diets inoculated with 10^5 $TCID_{50}$ of ASFV in liquid plasma for 14 consecutive days failed to cause disease.

Findings from the current study suggest that all feed matrices provided some level of protection to EhV. However, soybean-based ingredients and complete feed exhibited significantly greater protective effects on virus viability compared with responses in corn and corn co-products. When comparing the virus stability in stomach-only (pepsin hydrolysis) with combined stomach + small intestine (pepsin + pancreatin hydrolysis) conditions, no differences exceeding the estimated experimental error of the viability PCR method (±1 log) were observed. For Experiments II and III, the viability PCR method had an LOD of 2.5×10^3 viral particles due to inherent dilution effects and practical constraints preventing further scaling of the experiment. Therefore, viable EhV concentrations observed as numerically zero correspond to levels at or below the LOD, which is equivalent to a 3.4 log concentration. Matrices yielding negative results (zero viable EhV concentration) indicated a reduction in viral concentrations of at least the reported logarithmic units during the 24 h *in vitro* fermentation process.

4.3 Experiment III—assessment of EhV viability after *in vitro* large intestine fermentation

No differences were observed among treatments in EhV viability during the *in vitro* fermentation process. However, exact reductions in EhV concentrations could not be determined due to the low LOD of the viability PCR method of 2.5×10^3 viral particles due to inherent dilution effects and practical constraints preventing further scaling of the experiment. Therefore, viable

EhV concentrations observed as numerically zero correspond to levels at or below the LOD, which is equivalent to a 3.4 log concentration. Matrices yielding negative results (zero viable EhV concentration) indicated a reduction in viral concentrations of at least the reported logarithmic units during the 24 h *in vitro* fermentation process.

Based on an average reduction of 2.8 log units in EhV viability observed during stomach + small intestine digestion across all matrices, and an additional 3.8 log reduction during large intestine fermentation across all matrices, we estimate an average total reduction of about 6.7 log units during the entire total tract *in vitro* digestion and fermentation process. This reduction in average EhV viability ranged from 5.32 log units for solvent-extracted soybean meal to 7.47 log units for corn, which may be greater due to the LOD of the experiment. These results may explain differences in infectious doses among feed ingredient matrices reported by Niederwerder et al. (22) and Blázquez et al. (23). Differences in timing of virus release from feed ingredient matrices during the hydrolysis portion of the digestion process may partially explain the inconsistencies in ASFV infection when pigs consume infectious doses of contaminated feed.

5 Conclusion

Using EhV as a safe and suitable ASFV-like surrogate virus enables the conducting of challenging experiments to begin understanding the dynamics of ASFV survival and inactivation in various types of feed matrices under various conditions. Unlike results from previous studies, our results showed no appreciable viable virus inactivation in either corn- or soybean-based feed ingredients and complete feed when inoculated with 10^8 EhV/mL and stored at 4°C, 24°C, or 34°C for up to 120 days. Therefore, the use of extended storage time up to 120 days does not appear to be an effective mitigation practice against ASFV. We are also the first to report that between 5 to more than 7 log (99.999 to 99.999%) reductions in EhV in various feed matrices occur during the entire *in vitro* digestion and fermentation process. These reductions in EhV viability during the digestion process may be correlated with the ash concentrations in feed ingredient matrices, which may potentially explain inconsistencies in ASFV infection when pigs consume infectious doses of contaminated feed. Results from this initial study provided interesting new insights regarding the resiliency of EhV as a surrogate for ASFV in common feed matrices and simulated swine digestion and fermentation processes that will need to be confirmed by subsequent studies.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

GS: Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing. CR-C: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. PU: Conceptualization, Writing – review & editing. DS: Conceptualization, Formal analysis, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1498977/full#supplementary-material

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A triple protein-based ELISA for differential detection of ASFV antibodies

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African swine fever (ASF) caused by the ASF virus (ASFV) is a severe and highly contagious viral disease that poses a significant threat to the global pig industry. As no vaccines or effective drugs are available to aid prevention and control, early detection is crucial. The emergence of the low-virulence ASFV strain not expressing CD2v/MGFs (ASFVΔCD2v/ΔMGFs) has been identified domestically and internationally and has even become an epidemic in China, resulting in a complex epidemic. The commercialized ASFV ELISA kits available can detect the presence of ASFV infection in pigs, but they are unable to distinguish wild-type ASFV from gene-deleted strains. The current published ELISA assays can distinguish between the wild-type and CD2v gene-deleted ASFV but cannot differentiate wild-type and MGF505 gene-deleted ASFV or CD2v and MGF505 double-gene deleted ASFV infection, posing new challenges for an effective prevention and control of ASFV. In this study, the ASFV-p30, ASFV-CD2v, and ASFV-MGF505 proteins were expressed using a prokaryotic expression system, and a triple protein-based ELISA antibody detection method based on these proteins was successfully established to effectively differentiate between wild-type ASFV and ASFVΔCD2v and/or ASFVΔMGF505 virus infection. This triple protein-based ELISA showed good analytical specificity without cross-reactivity with antibodies against PRRSV, CSFV, PRV, and PCV2. Moreover, it demonstrates remarkable analytical sensitivity by allowing the identification of clinical samples even at dilutions as high as 1:800. The coefficient of variation the intra-assay and inter-assay were below 5%, indicating strong repeatability and reproducibility. To evaluate the performance of the triple protein-based ELISA, a total of 59 clinical serum samples were detected using the triple protein-based ELISA. The results showed that 22 samples were positive for ASFV, of which 19 were ASFV wild-type, one was ASFVΔCD2v, and two were ASFVΔMGF505. Compared with the commercialized triplex qPCR kit, the triple protein-based ELISA exhibited high diagnostic sensitivity and diagnostic specificity. The test accuracy with the commercialized triplex qPCR kit was 98.31% (58/59), and the test accuracy with the commercialized ELISA kit was 96.61% (57/59). These results indicated that the developed triple protein-based ELISA performs well in detection and differentiation. Therefore, it will be useful for the ASFV serological differential diagnosis and epidemiology study.

KEYWORDS

African swine fever virus, indirect ELISA, differential diagnosis, wild-type strain, gene-deleted strain

1 Introduction

African swine fever (ASF) is a highly contagious infectious disease caused by the ASF virus (ASFV) (1). Clinical manifestations range from acute to chronic or asymptomatic (2, 3). Acute ASF caused by highly virulent ASFV (usually named wild-type ASFV), has a fatality rate of 100% (3, 4). Chronic or asymptomatic ASF caused by lower virulent mutant are highly transmissible, can shed ASFV for a long time and infect susceptible pigs via direct or indirect contact (5–8), but not easy to be find clinically. ASFV was first discovered in Kenya in 1921 and has spread to many countries (9–15). Since ASF was introduced in China in August 2018, it quickly spread across pig farms throughout China and led to a sharp decline in pig populations (16). From the end of 2020, naturally attenuated ASFV strains emerged in some domestic pig farms in China and some other ASFV affected countries (17–23). The co-circulating of the wild type ASFV and naturally lower-virulence ASFV complicates the situation of epidemic disease. According to the WOAH reported from January 2020 to January 2022, 35 countries have reported African swine fever outbreaks, resulting in significant losses in domestic pig (1,043,334 animals lost) and wild boar populations (29,970 animals lost) (24). Currently, ASF has spread extensively globally, brings significant economic losses to the pig industry.

The etiological agent, ASFV, is a large double-stranded DNA virus with an envelope and icosahedral structure. The full length of the ASFV DNA genome varies between 170 kb and 193 kb. It encodes about 200 proteins, most of which are related to virus replication, immune escape, virus transmission, and so on (15). The p30 protein encoded by the CP204L gene is a relatively conserved immunogenic capsid protein of ASFV, which can stimulate the body to produce antibodies with certain neutralizing abilities in the early stage of virus infection and last for a long time. It is an ideal antibody diagnostic antigen for ASFV (25, 26). CD2v protein is expressed in the late stage of ASFV infection and encoded by the EP402R gene. Wild-type ASFV infection induces the production of specific antibodies that recognize the CD2v protein. CD2v protein is an important structural surface antigen of wild-type ASFV and the key protein to distinguish wild-type ASFV from CD2v gene-deleted ASFV. It is related to the pathogenicity of the virus. Compared with wild-type ASFV virulent strains, the time of viremia after the weak strain of CD2v knockout infecting the host is prolonged, and clinical symptoms are also reduced. In addition, CD2v can inhibit lymphocyte proliferation and induce immunosuppression (17, 27). In the family of MGF genes, the MGF505 gene is involved in immune escape, can target TANK binding kinase 1 (TBK1), inhibit cGAS-STING-mediated IFN-β production, and is an important structural protein for ASFV invasion (28). Partial deletion of the MGF505 gene can reduce viral replication and virulence in alveolar macrophages (1). Therefore, CD2v and MGFs are usually used as targets to design attenuated vaccines and develop diagnostic assays.

Vaccines are always the most effective preventive tools against viral diseases. Since the outbreak of ASF, various approaches have been employed in ASF vaccine design. According to the currently available information, gene deleted live attenuated vaccines (LAVs) generated by rational deleting a single or multiple virulence genes appear to be the most promising vaccine candidates and exhibit a wide range of safety and efficacy against ASF (29–31). Currently, several gene-deleted LAV vaccine candidates have been generated, such as those with the deletion of the CD2v, UK, MGFs and DP148R genes and so on in the ASFV genome (1, 32). However, except for Vietnam, where live attenuated vaccines ASFV-G-ΔI177L with deletion of the I177L gene and ASFV-G-ΔMGF with deletion of six genes: MGF505-1R, MGF360-12L, MGF360-13L, MGF360-14L, MGF505-2R, and MGF505-3R have been recently commercialized, no registered vaccines are available to prevent and control ASF in other countries (1, 30, 33, 34). Therefore, rapid and specific molecular diagnosis and serological detection by PCR and ELISA, recommended by WOAH, play a pivotal role in preventing and controlling ASF (1). However, naturally gene-deleted lower-virulence ASFV (ASFVΔCD2v/ΔMGF) identified in China and some other country generally cause a chronic and persistent infection course in pigs, lacking typical clinical symptoms compared with wild-type ASFV infections (2, 14, 17–19, 21, 27). The delayed epidemic progression caused by these low-virulent strains results in a postponement or intermittent virus-shedding process in oral and nasal secretions, and the virus is generally undetectable in the blood as well as in nasal and oral secretions after viremia (2, 4, 14). Therefore, ASFV infection cannot be reliably monitored by detecting the virus alone. Regarding antibody detection, the commercial ASFV ELISA kits available can only detect antibody levels in a sample but cannot differentiate serum-positive pigs from wild-type ASFV infection or lower-virulence ASFV with gene deletion. The current published ELISA assays can distinguish between the wild-type and natural CD2v gene-deleted ASFV, but cannot differentiate wild-type and MGF505 gene-deleted ASFV or CD2v and MGF505 double gene-deleted ASFV infection. Therefore, they cannot fully meet the requirements to monitor the current epidemic variation of ASFV. In addition, although there has no commercial vaccine in China, several studies have confirmed that the most potential ASF vaccine candidates are CD2v and MGFs gene-deleted live attenuated vaccines (8, 14, 35, 36). Among these candidates, HLJ/18-7GD with the deletion of seven genes encoding MGF5051R, MGF505-2R, MGF505-3R, MGF360-12L, MGF36013L, MGF360-14L and CD2v, have undergone clinical trials and demonstrated promising potential for vaccine development (29, 37). Therefore, it is imperative to establish an ELISA antibody detection method that can effectively distinguish wild-type ASFV and CD2v and/or MGFs gene-deleted strains.

In this study, the ASFV p30, CD2v, and MGF505 proteins were expressed and purified. A highly sensitive and specific triple protein-based ELISA was developed based on these proteins to detect ASFV antibodies. This newly established ELISA antibody assay can rapidly detect ASFV infection and differentiate the causative virus from wild-type ASFV or CD2v and/or MGF505 gene-deleted strains infection. It offers technical support for the differential diagnosis, epidemiological study of ASFV, and vaccine effectiveness evaluation in the future.

2 Materials and methods

2.1 Serum samples

Standard ASFV negative ($ASFV^-$) and ASFV positive ($ASFV^+$) sera were obtained from Qingdao Lijian Bio-tech Co. Ltd. (Qingdao, China) and are used to test the optimal concentration of antigen coating and serum dilution; CD2v unexpressed ASFV ($ASFV^+$ΔCD2v)

positive sera and MGF unexpressed ASFV (ASFV$^+$ΔMGF505) positive sera were stored at −80°C in our laboratory and are used to identify the immunogenicity of the proteins ASFV-p30, ASFV-CD2v and ASFV-MGF505. These sera were collected from pigs which were infected by ASFV$^+$ΔCD2v or ASFV$^+$ΔMGF505 virus strains. The infection status of these pigs was confirmed by detecting ASFV DNA using a commercialized triplex qPCR kit based on p72/CD2v/MGF gene (BioKITai, Xiamen, China) and the serum samples were collected 4 weeks after ASFV infection was verified. Serum samples collected from Pig farms before 2018 ($n = 30$) and from pig farms with no history of ASFV infection from 2019 to 2023 ($n = 60$) are used to determine the threshold value of the assay. These sera were confirmed to be negative for ASFV by a commercialized triplex qPCR kit based on p72/CD2v/MGF gene (BioKITai, Xiamen, China) and a commercialized ELISA kit based on p30 protein (Putai Biology, Luoyang, China). Antibody-positive sera of porcine reproductive and respiratory syndrome virus (PRRSV$^+$), porcine pseudorabies virus (PRV$^+$), porcine circovirus type 2 (PCV2$^+$) and classical swine fever virus (CSFV$^+$) were confirmed to be positive by the commercialized ELISA kits (Putai Biology, Luoyang, China) and to be negative for ASFV by a commercialized ELISA kit based on p30 protein (Putai Biology, Luoyang, China) were stored at −80°C in our laboratory. These sera samples were used to validate the analytical specificity of the triple protein-based ELISA. Clinical serum samples ($n = 59$) used to evaluate the precision and test accuracy of ELISA methods with commercially available kits were collected from pig farms suspected of ASFV infection in northern China (including Inner Mongolia Autonomous Region, Shanxi Province, Hebei Province, Shandong Province, etc.) in 2020–2023. These samples were tested by a commercialized triplex qPCR kit based on p72/CD2v/MGF gene (BioKITai, Xiamen, China) and showed 23 ASFV$^+$ serum (21 ASFV$^+$ wild-type serum, 1 ASFV$^+$ΔCD2v serum, 1 ASFV$^+$ΔMGF505 serum), and 36 ASFV$^-$ serum. These samples were also detected using a commercialized ELISA kit based on p30 protein (Putai biology, Luoyang, China) and showed 24 ASFV$^+$ serum, 35 ASFV$^-$ serum.

2.2 Prokaryotic expression, purification, and immunogenicity identification of recombinant proteins ASFV-p30, ASFV-CD2v, and ASFV-MGF505

The sequences of ASFV p30, CD2v, and MGF505 (ASFV Pig/HLJ/2018, GenBank: MK333180.1) were synthesized by Sangon Biotech (Shanghai, China) and separately cloned into the pET-32a vector through the *EcoR I* and *Xho I* restriction sites (TaKaRa, China). The recombinant plasmids were transformed into competent *Escherichia coli* (*E. coli*) BL21 (DE3) cells after sequence identification and cultured at 37°C. The negative control strains (pET-32a plasmids transformed BL21 cells) were cultured simultaneously. When the culture OD_{450nm} reached 0.7, isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5, 1, 1.5, 2%) (Solarbio, Beijing, China) was added to induce protein expression for 2–8 h to optimize protein expression conditions. These proteins were purified by the His-labeled protein purification kit (CWBIO, Jiangsu, China), according to the manufacturer's instructions.

The endotoxins in purified proteins was removed using a ToxinEraser Endotoxin Removal Kit (Genscript, Nanjing, China) and was detected using a ToxinSensor Chromogenic LAL Endotoxin Assay Kit (Genscript, Nanjing, China) following the manufacturer's instructions to determine. SDS-PAGE and NanoDrop 2000 were used to analyze the concentration and purity of the purified recombinant proteins ASFV-p30, ASFV-CD2v, and ASFV-MGF505. The yield of expressed proteins was calculated according to the concentration of recombinant proteins. Western blot was used to analyze their antigenicity. Following SDS-PAGE, these proteins were transferred to the PVDF membranes. After overnight sealing with 5% skim milk, the PVDF membranes were incubated with sera against ASFV$^+$ wild-type, ASFV$^+$ΔCD2v, or ASFV$^+$ΔMGF505 (1:2,000) for 3 h. Then, the PVDF membranes were incubated with HRP labeled Rabbit Anti-Pig IgG (H + L) (Biodragon, Beijing, China) (1:5,000) for 3 h. Results were shown by the eECL Western Blot Kit (CWBIO, Jiangsu, China).

2.3 Establishment of triple protein-based ELISA method based on recombinant protein ASFV-p30, ASFV-CD2v, and ASFV-MGF505

The purified recombinant proteins ASFV-p30, ASFV-CD2v, and ASFV-MGF505 were used as coating antigens to establish a triple protein-based ELISA to differentiate serum antibodies between wild-type and CD2v /MGF505 gene-deleted ASFV infection. ELISA was carried out on 96-well microtiter plates (BIOFIL, Guangzhou, China). A checkerboard titration of each antigen pool and serum was designed to determine the optimal dilutions of the antigen and serum. The purified recombinant proteins ASFV-p30, ASFV-CD2v, and ASFV-MGF505 were diluted using carbonate buffer (0.159% (w/v) Na_2CO_3; 0.293% (w/v) $NaHCO_3$) with the range of 4 to 0.125 μg/mL. Microtiter plates were coated with 100 μL per well of the diluted protein and incubated for 1 h at 37°C, then overnight at 4°C. After washed five times with PBST (Solarbio, Beijing, China), the plates were blocked for 3 h at 37°C with 5% skim milk in PBST (Solarbio, Beijing, China) (100 μL/well). Then, the plates were washed as described above, and 100 μL of the serum samples (antibody-positive and-negative standard sera control) that were diluted (from 1:50 to 1:400) with 5% skim milk in PBST was added and incubated for 1 h at 37°C. Subsequently, the 96-well plates were washed and incubated with 100 μL of a 1:5,000 dilution of horseradish peroxidase-conjugated Rabbit Anti-Pig IgG (H + L) per well for 1 h at 37°C. After being washed five times with PBST, the reaction was visualized by incubating the wells with a TMB single component substrate solution (Solarbio, Beijing, China) (100 μL/well) for 10 min at 37°C. The reaction was stopped by adding 50 μL 2 M H_2SO_4 per well and the optical density (OD) at 450 nm of each well was measured immediately using an enzyme labeler (Thermo Fisher Scientific, Multiskan FC). All samples were run in triplicate. The optimal antigen and serum concentrations were determined using the criteria that the $OD_{450\,nm}$ value of the positive serum was closest to 1.0, together with a maximum positive/negative (P/N) value.

When only the type of blocking solution, the time and temperature of antigen coating, or the dilution concentration of HRP labeled Rabbit Anti-Pig IgG (H + L) were used as the single variable of the ELISA condition, and other steps were the same as above, the optimal blocking solution, antigen coating time and temperature, or the dilution concentration of ELISA were tested. As previously described,

the optimal assay conditions were identified as those that yielded the highest P/N value and the ASFV$^+$ $OD_{450\,nm}$ value closest to 1.0.

2.4 Confirmation of the cut-off value and the result criterion

The cut-off value was determined by detecting ASFV$^-$ serum samples under the optimal test conditions. The status of these serum samples was confirmed by a commercialized triplex qPCR kit based on p72/CD2v/MGF gene (BioKITai, Xiamen, China) and a commercialized ELISA kit (Putai Biology, Luoyang, China). The $OD_{450\,nm}$ value of these ASFV negative serum samples obtained with p30-ELISA, CD2v-ELISA, and MGF505-ELISA was recorded. The cut-off value of each ELISA was established at the mean $OD_{450\,nm}$ value ($\bar{x}$) + 3 × the standard deviation (SD). The serum sample was considered positive if the $OD_{450\,nm}$ value exceeded the cut-off value. Otherwise, it was considered negative. The criteria to determine the triple protein-based ELISA results are presented in Table 1.

2.5 Evaluation of analytical specificity, analytical sensitivity, repeatability and reproducibility

Serum samples of ASFV$^-$, PRRSV$^+$, PRV$^+$, CSFV$^+$, PCV2$^+$, standard ASFV$^+$, ASFV$^+$ΔCD2v, and ASFV$^+$ΔMGF505 were detected to determine the analytical Specificity of the developed ELISA at 1:100 dilution. The analytical Sensitivity of the triple protein-based ELISA was evaluated using the standard ASFV$^+$ serum diluted from 1: 100 to 1: 3,200. ASFV positive serum samples were tested in the same batch to evaluate the repeatability (intra-assay) of the developed ELISA and were tested at different laboratories to evaluate the reproducibility (inter-assay) of the triple protein-based ELISA.

2.6 Comparison with the commercialized kit

A total of 59 clinical serum samples were detected using a commercialized triplex qPCR kit based on p72/CD2v/MGF gene and a commercialized ELISA kit based on p30 protein for a comparison and as a reference method as well. The clinical serum samples were detected using the developed triple protein-based ELISA to calculate the relative diagnostic sensitivity, diagnostic specificity and test accuracy of triple protein-based ELISA method. In this study, we defined relative diagnostic sensitivity (TP/ (TP + FN)) as the ratio of positive tests from the established ELISA to the positive tests by the commercialized triplex qPCR kit, and relative diagnostic specificity (TN/(TN + FP)) was defined as the ratio of negative tests from the established ELISA to the commercialized triplex qPCR kit. Test accuracy = (TP + TN)/(TP + FN + FP + TN) × 100% (Table 2).

TABLE 1 Criteria for the triple protein-based ELISA results.

$OD_{450\,nm}$ of p30/CD2v/MGF505 protein-coated well			Determination of results
p30 (>cut-off value)	CD2v (>cut-off value)	MGF505 (>cut-off value)	
Y	Y	Y	ASFV$^+$ wild-type
Y	N	Y	ASFV$^+$ΔCD2v
Y	Y	N	ASFV$^+$ΔMGF505
Y	N	N	ASFV$^+$ΔCD2v and ΔMGF505
N	N	N	ASFV$^-$

"Y" indicates the $OD_{450\,nm}$ of serum samples greater than the cut-off value, and "N" indicates the $OD_{450\,nm}$ of serum samples less than the cut-off value.

2.7 Statistical analysis

All experiments were repeated at least three times. SPSS (IBM Corporation, Armonk, NY, USA) and Origin 8.0 (OriginLab, Northampton, MA, USA) were used to perform all statistical tests. All data were presented as the mean + SD. Statistical significance was analyzed using the t-test and was considered at $p < 0.05$.

3 Results

3.1 Prokaryotic expression, purification, and immunogenicity identification of recombinant proteins ASFV-p30, ASFV-CD2v, and ASFV-MGF505

The synthesized protein genes ASFV-p30, ASFV-CD2v, and ASFV-MGF505 were separately cloned into the pET-32a vector and successfully expressed in *E. coli* BL21 cells with the highest expression level under the condition of 1% IPTG induction for 6 h. The calculated molecular weights of the recombinant proteins were 39.6 kDa (Figure 1A), 46.8 kDa (Figure 1B), and 42.3 kDa (Figure 1C), respectively. After purification, highly-purity proteins were obtained. The yield of recombinant Proteins ASFV-p30, ASFV-CD2v, and ASFV-MGF505 was calculated to be 15.28 mg, 11.34 mg, 11.09 mg in 100 mL LB medium. The endotoxins content of these proteins in this study did not exceed 0.1 EU/mg. Western blot analysis was applied to identify purified ASFV-p30, ASFV-CD2v, and ASFV-MGF505. All three recombinant proteins could react with ASFV$^+$ sera (Figure 1D) but not with ASFV$^-$ sera (Figure 1G). ASFV-p30 and ASFV-CD2v proteins could be recognized by ASFV$^+$ΔMGF505 serum, but ASFV-MGF505 protein could not (Figure 1E). ASFV-p30 and ASFV-MGF505 proteins could react with ASFV$^+$ΔCD2v serum, but ASFV-CD2v protein could not (Figure 1F). These results indicated that the purified ASFV-p30, ASFV-CD2v, and ASFV-MGF505 proteins had high immunogenicity, and that anti-ASFV-CD2v and anti-MGF505 antibodies present in sera do not cross-react with ASFV-MGF505 and ASFV-CD2v, respectively.

3.2 Optimization of experimental conditions for the triple protein-based ELISA

The optimal antigen concentration and serum sample dilution were determined using checkerboard titration. The maximum value of P/N was obtained when the concentration of the ASFV-p30 and

TABLE 2 Calculation of test accuracy between triple protein-based ELISA and commercialized qPCR/ELISA kit.

	The triple protein-based ELISA (+)	The triple protein-based ELISA (−)	Total
The commercialized qPCR/ELISA kit (+)	TP	FN	TP + FN
The commercialized qPCR/ELISA kit (−)	FP	TN	FP + TN
Total	TP + FP	FN + TN	TP + FN + FP + TN

TP, the number of the true positive samples; TN, the number of the true negative samples. FP, the number of the false positive samples; FN, the number of the false negative samples.

ASFV-CD2v protein was 0.5 μg/mL, ASFV-MGF505 protein was 1 μg/mL (Figure 2A) and the serum dilution was 1:100 (Figure 2B). The optimal antigen coating time and temperature was 37°C for 1 h and then overnight at 4°C (Figure 2C). The best blocking buffer was PBST with 5% skim milk (Figure 2D), and the optimal dilution of HRP labeled Rabbit Anti-Pig IgG (H + L) was 1:10,000 (Figure 2E).

3.3 Confirmation of the cut-off value

The cut-off value was determined by detecting serum samples negative for ASFV under optimal conditions. The mean $OD_{450\,nm}$ value of ELISA based on the protein ASFV-p30, ASFV-CD2v, and ASFV-MGF505 was 0.219, 0.190, and 0.176, with an SD of 0.038, 0.041, and 0.057, respectively. Therefore, the cut-off value of the ELISA based on the protein ASFV-p30, ASFV-CD2v, and ASFV-MGF505 was calculated to be 0.333, 0.313, and 0.347 (mean $OD_{450\,nm}$ value of negative samples plus three SD), respectively (Figure 3).

3.4 Evaluation of analytical specificity, analytical sensitivity, repeatability and reproducibility

The analytical specificity of the triple protein-based ELISA method was evaluated by detecting the reactivity of each purified protein with antibodies against $ASFV^{-}$, $PRRSV^{+}$, PRV^{+}, $CSFV^{+}$, $PCV2^{+}$, standard $ASFV^{+}$, $ASFV^{+}\Delta CD2v$, and $ASFV^{+}\Delta MGF505$. Only the standard $ASFV^{+}$ (the mean $OD_{450\,nm}$ ± SD: 1.216 ± 0.007, 1.154 ± 0.044), $ASFV^{+}\Delta CD2v$ (the mean $OD_{450\,nm}$ ± SD: 1.208 ± 0.014), and $ASFV^{+}\Delta MGF505$ (the mean $OD_{450\,nm}$ ± SD: 1.041 ± 0.054) serum samples were positive in ASFV-p30 protein-coated ELISA; standard $ASFV^{+}$ (the mean $OD_{450\,nm}$ ± SD: 1.101 ± 0.014, 0.947 ± 0.043) and $ASFV^{+}\Delta MGF505$ (the mean $OD_{450\,nm}$ ± SD: 1.129 ± 0.012) were positive in ASFV-CD2v protein-coated ELISA; and standard $ASFV^{+}$(the mean $OD_{450\,nm}$ ± SD: 1.273 ± 0.054, 1.583 ± 0.069) and $ASFV^{+}\Delta CD2v$ (the mean $OD_{450\,nm}$ ± SD: 0.991 ± 0.059) serum samples were positive in ASFV-MGF505 protein-coated wells. On the contrary, $ASFV^{-}$ serum samples and other pathogen positive sera were negative in the triple ELISA. Overall, the triple protein-based ELISA assays were an effective method for detecting and distinguishing ASFV antibodies (Figure 4A).

The analytical sensitivity of the triple protein-based ELISA method was assessed by detecting serial dilutions of standard $ASFV^{+}$ serum samples. Both the ASFV-p30 and ASFV-CD2v protein-based ELISA could detect a 1,600-fold dilution of standard $ASFV^{+}$ serum, while the ELISA method based on the ASFV-MGF505 protein could detect an 800-fold dilution of standard $ASFV^{+}$ serum, revealing that the detection limit of the triple ELISA was 1: 800 (Figure 4B).

ASFV positive serum samples were analyzed to validate the repeatability and reproducibility of the triple protein-based ELISA method. The CV values of intra-assay and inter-assay were below 5% (Table 3), indicating the good repeatability and reproducibility of the triple protein-based ELISA.

3.5 Comparison with the commercialized kit

Clinical serum samples (n = 59) were tested by a commercialized triplex qPCR kit, commercialized ELISA kit and the triple protein-based ELISA to calculate the diagnostic sensitivity, diagnostic specificity and test accuracy of established triple protein-based ELISA method. The triple protein-based ELISA identified a total of 22 $ASFV^{+}$ sera (19 $ASFV^{+}$ wild-type sera, 1 $ASFV^{+}\Delta CD2v$ serum, 2 $ASFV^{+}\Delta MGF505$ sera), and 37 $ASFV^{-}$ sera (Figure 5). Compared with the commercialized triplex qPCR kit, the relative diagnostic sensitivity of the triple protein-based ELISA based on the protein ASFV-p30 was 95.65% (95% confidence interval: 78.05 to 99.89%) among the $ASFV^{+}$ serum, and the relative diagnostic specificity of the method was 97.30% (95% confidence interval: 85.84 to 99.93%) among the $ASFV^{-}$ serum; the relative diagnostic sensitivity of the triple protein-based ELISA based on the protein ASFV-CD2v was 95.45% (95% confidence interval: 77.16 to 99.89%) among the $ASFV^{+}$ serum, and the relative diagnostic specificity of the method was 97.37% (95% confidence interval: 86.19–99.93%) among the $ASFV^{-}$ serum; the relative diagnostic sensitivity of the triple protein-based ELISA based on the protein ASFV-MGF505 was 90.91% (95% confidence interval: 70.84–98.88%) among the $ASFV^{+}$ serum, and the relative diagnostic specificity of the method was 94.87% (95% confidence interval: 82.68–99.37%) among the $ASFV^{-}$ serum. The test accuracy of the triple protein-based ELISA and commercialized triplex qPCR kit was 98.31% (58/59). Compared with the commercialized ELISA kit, the test accuracy was 96.61% (57/59). These results indicating that the method can diagnose ASF and differentiate serum antibodies from wild-type ASFV from CD2v/MGF505 unexpressed ASFV infection (Table 4). Compared with the commercialized ELISA kit available and published ELISA methods, the triple protein-based method overcomed the current limitation of not being able to distinguish wild-type and MGF505 gene-deleted ASFV or CD2v and MGF505 double gene-deleted ASFV infection. It is of great significance to distinguish ASFV infected strains in pig farms and take corresponding prevention and control measures.

4 Discussion

ASFV is progressively spreading throughout the world, which has substantial economic implications for the global pig industry (14). The emergence of gene-deleted ASFV strains characterized by longer incubation and high transmission ability increased the difficulty of ASF control (27). Because there are no effective anti-ASFV drugs and commercialized ASFV vaccines available, cutting off transmission

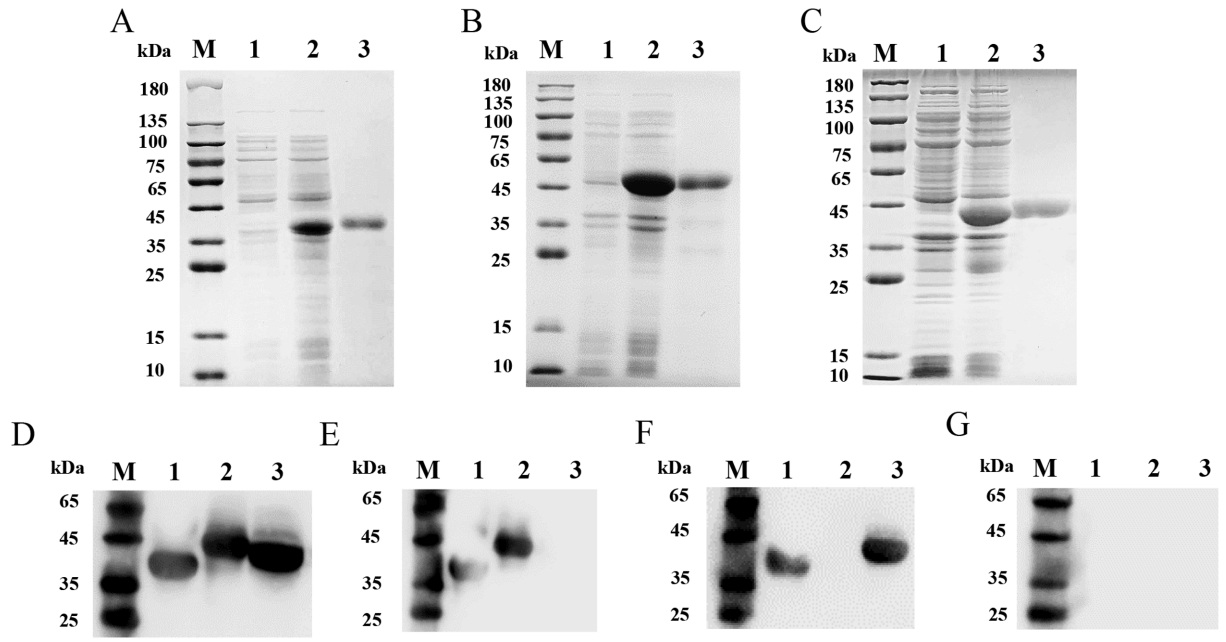

FIGURE 1
SDS-PAGE and Western blot analysis of purified recombinant proteins ASFV-p30, ASFV-CD2v, and ASFV-MGF505. SDS-PAGE analysis of purified recombinant proteins ASFV-p30, ASFV-CD2v, and ASFV-MGF505 **(A–C)**. The expression and purification of the recombinant proteins ASFV-p30 (39.6 kDa) **(A)**, ASFV-CD2v (46.8 kDa) **(B)**, and ASFV-MGF505 (42.3 kDa) **(C)** were analyzed by SDS-PAGE. Lane M: protein marker; Lane 1: pET-32a; Lane 2: unpurified protein; Lane 3: purified protein. Analysis of immunogenic identification of the ASFV-p30, ASFV-CD2v, and ASFV-MGF505 proteins by Western blot assay **(D–G)**. ASFV-p30, ASFV-CD2v, and ASFV-MGF505 proteins could react with ASFV^{+} serum **(D)**. ASFV-p30 and ASFV-CD2v proteins could react with ASFV^{+}ΔMGF505 serum **(E)**. ASFV-p30 and ASFV-MGF505 proteins could react with ASFV^{+}ΔCD2v serum **(F)**. ASFV-p30, ASFV-CD2v, and ASFV-MGF505 proteins could not react with ASFV^{-} serum **(G)**. Lane M: protein marker; Lane 1: ASFV-p30; Lane 2: ASFV-CD2v; Lane 3: ASFV-MGF505.

routes by strict biosecurity measures, together with effective detection for early diagnosis and elimination of infected pigs, are currently the primary strategy for prevention and control (3, 4, 38).

Many methods have been developed to diagnose ASFV infection by detecting ASFV or ASFV-specific antibodies. Among these detection methods, fluorescence quantitative PCR (qPCR) and ELISA are considered the main methods owing to the advantages of high throughput, specificity, and sensitivity (38). For the detection of ASFV, several single or multiplex qPCRs based on different genes have been reported, except for commercialized qPCR kits (39, 40). All these reported detection methods can effectively detect ASFV in positive samples. In addition, some assays can also differentially detect wild-type and gene-deleted ASFV (39, 40). However, the postponed or discontinuous virus shedding process caused by the gene-deleted low-virulent strains which often result in false negative detection results in some ASFV infected cases (4). In addition, ASF was classified as a first-class animal infectious disease in China (4). These molecular diagnostic methods, which use ASFV nucleic acid as the detected target, have stringent requirements for biosafety facilities, diagnostic personnel, and the viral genome extraction process (4, 38, 41). Compared with qPCR, ELISA does not have strict requirements because there is no need to handle viruses. Furthermore, in the absence of a vaccine, specific antibodies in the serum indicate virus infection, and these antibodies can persist for a long time in infected pigs (4, 15, 41, 42). Therefore, antibody detection by serological assays is essential for the prevention and control of ASF, especially for subacute and chronic ASF (15).

Currently, using p30, p32, p54, p62, p72, and other viral proteins, several single, double, triple, or quadruple antigen-based ELISA assays were established for the detection of ASFV antibodies, and some ELISA kits have been commercialized (15, 16, 25, 38, 43–45). These methods can accurately distinguish the serum-positive pig from the negative one but cannot differentiate the serum-positive pigs infected with wild-type or gene-deleted ASFV. To identify wild-type and CD2v-deleted strains of ASFV, an indirect ELISA based on the extracellular domain of the expressed CD2v protein has been established (17). This method does not have cross-reaction with serum samples infected with CD2v deleted ASFV, PRRSV, CSFV, PCV, PRV, swine FMDV, and PEDV. The serum dilution that can be identified was 1:2,560. The coefficient of variation in and between batches was <10%, and the compliance rate was 99.4% (17). However, this indirect ELISA only uses the extracellular domain of the CD2v protein as the detection protein. When the detected result is negative, it is hard to determine whether the pig was not infected with ASFV or infected with CD2v gene-deleted ASFV using this ELISA method alone. To meet this requirement, this assay should be used in combination with traditional ELISA simultaneously. In order to differentiate wild-type and CD2v gene-deleted ASFV, a dual ELISA based p30 and CD2v protein was established (14). The dual ELISA showed excellent specificity without cross-reactions with antibodies of PRRSV, CSFV, JEV, PRV, or PPV, and high sensitivity with a maximum detect dilution of the ASFV-infected positive serum samples of 5,120 times (14). Another dual indirect ELISA based on p54 and CD2v proteins has been developed to specifically distinguish

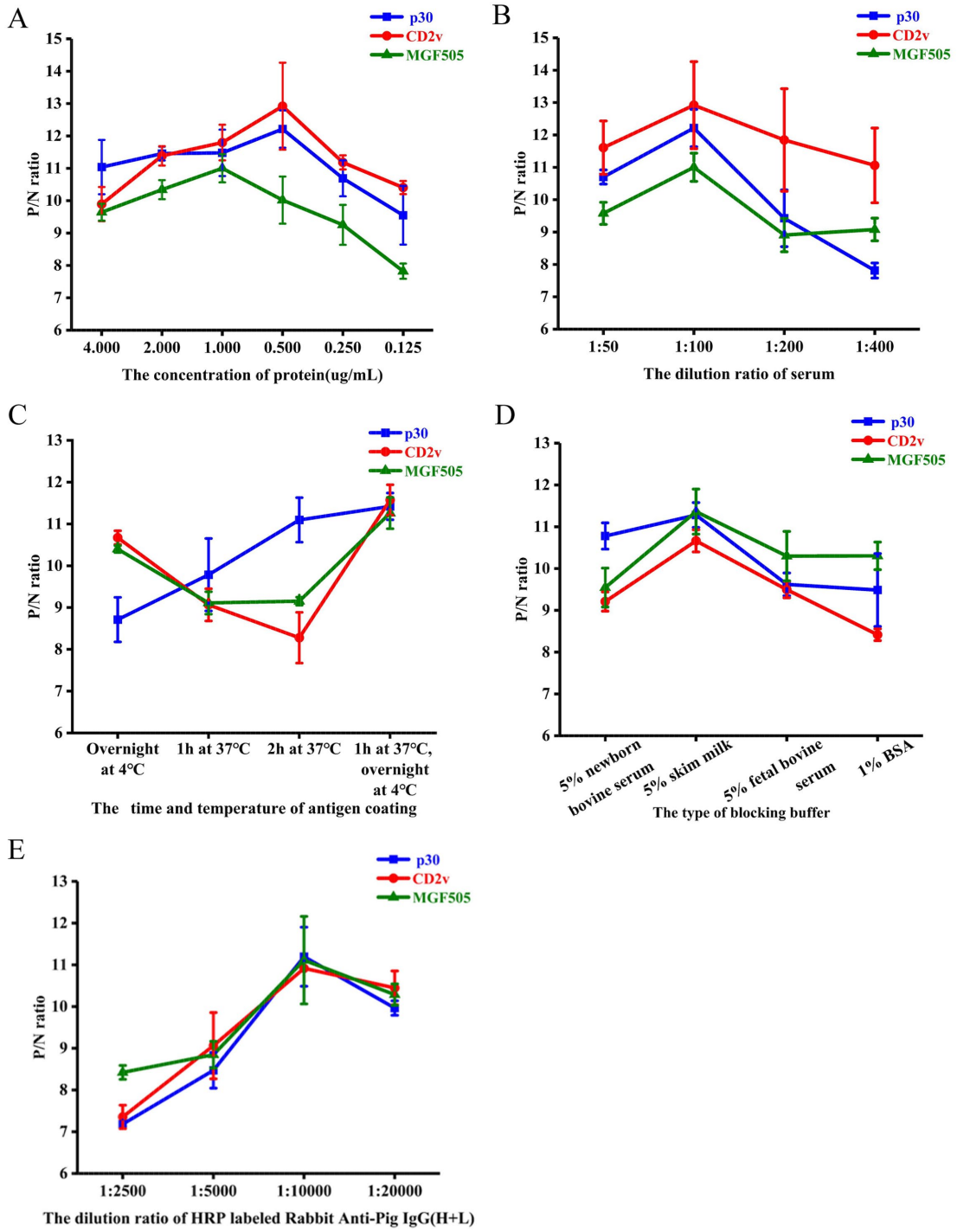

FIGURE 2
Optimization of experimental conditions for the triple protein-based ELISA. Determination of optimal protein coating concentration. P/N ratios of an ASFV$^+$ and ASFV$^-$ serum using different concentrations of the ASFV-p30, ASFV-CD2v, and ASFV-MGF505 proteins **(A)**. Determination of optimal serum dilution. P/N ratios of an ASFV$^+$ and ASFV$^-$ serum with various dilutions of serum samples **(B)**. Determination of optimal antigen coating conditions. P/N ratios of an ASFV$^+$ and ASFV$^-$ serum with various coating times and temperatures of each antigen **(C)**. Determination of the best blocking buffer. P/N ratios of an ASFV$^+$ and ASFV$^-$ serum with various blocking buffer **(D)**. Determination of the optimal dilution of the HRP labeled Rabbit Anti-Pig IgG (H + L). P/N ratios of an ASFV$^+$ and ASFV$^-$ serum with various dilutions of HRP labeled Rabbit Anti-Pig IgG (H + L) **(E)**. P/N ratio data represent mean ± SD.

serum antibodies from pigs infected with wild-type ASFV or possessing attenuated vaccine candidate HLJ/18-7GD immunization (42). The results showed that the diagnostic method has excellent specificity and good reproducibility. It can not only effectively distinguish antibodies induced by wild-type virulent ASFV infection from the vaccine candidate HLJ/18-7GD immunization, but also appropriate to differentiate wild-type and natural CD2v gene-deleted ASFV infection (42). Recently, two ASFV-p72 and -CD2v

nanobody-based competitive ELISAs (cELISAs) were developed to detect anti-ASFV antibodies. The two assays showed high sensitivity, specificity, reproducibility, and stability and their combination could differentiate pigs infected with wild-type and CD2v-deleted ASFV (36). However, although these dual ELISA methods have high specificity and sensitivity and are appropriate for differentiating a wild-type and CD2v deleted ASFV infection, they cannot differentiate wild-type and MGF505 gene-deleted ASFV or CD2v and MGF505 double-gene-deleted ASFV infection. Furthermore, they cannot distinguish MGF505 gene-deleted ASFV from CD2v and MGF505 double-gene-deleted ASFV.

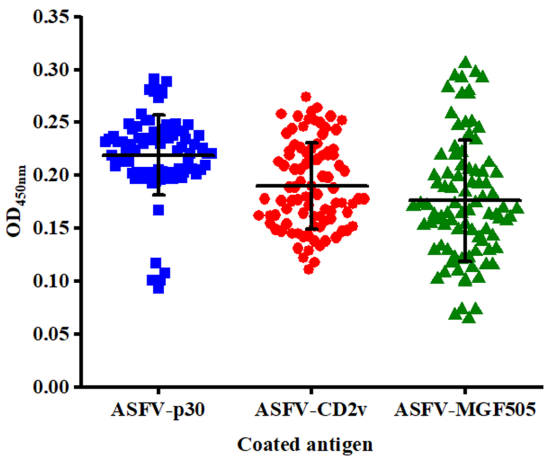

FIGURE 3
Confirmation of the cut-off value for the triple protein-based ELISA. ASFV⁻ serum samples (n = 90) were tested by the triple protein-based ELISA to determine the cut-off value under the optimal test conditions. The cut-off value of each ELISA was established at the mean $OD_{450\,nm}$ value ($\bar{x}$) + 3 × the standard deviation (SD).

The ASFV p30 protein is a crucial structural component that exhibits early expression and phosphorylation in infected cells, showing robust immunogenicity. The p30 protein was reported to be one of the most antigenic ASFV proteins and is generally used as an antigen to develop a serological diagnosis (38, 46). The ELISA detection method using p30 as the coating antigen can basically be used for the whole process of monitoring after ASFV infection (25) and some have been commercialized, such as the commercial ELISA kits (Svanovir, Uppsala, Sweden) and (Putai biology, Luoyang, China).

This study expressed the proteins ASFV-p30, ASFV-CD2v, and ASFV-MGF505 in *E. coli* and purified these proteins by the His-labeled protein purification kit. After purity testing and endotoxin removal, using these proteins as coating antigens, a triple protein-based ELISA assay was developed. The triple protein-based ELISA realized the differentiation detection of different ASFV infection strains by coating p30 protein expressed by all ASFV strains, CD2V protein not expressed in ASFV⁺ΔCD2v strains, and MGF505 protein not expressed in ASFV⁺ΔMGF505 strains. The assay overcomed the limitations of the commercialized ELISA kit available and published ELISA methods that cannot distinguish MGF505 gene-deleted ASFV from the wild-type, and the CD2v and MGF505 double-gene-deleted ASFV. The steps of recombinant protein purification and endotoxin removal procedure mitigated the potential contamination with bacterial proteins and endotoxins due to the use of a prokaryotic expression system (*E. coli*), thus reduced the false-positive results of the developed ELISA. It was known that the dilution of testing sera, the reaction regents and reaction time of each process are usually different between different ELISA kits, each need a separately operation which was complex and time consuming. In this study, although each protein of this triple ELISA is independent detection of ASFV virus antibodies against the corresponding proteins, the optimal reaction conditions of

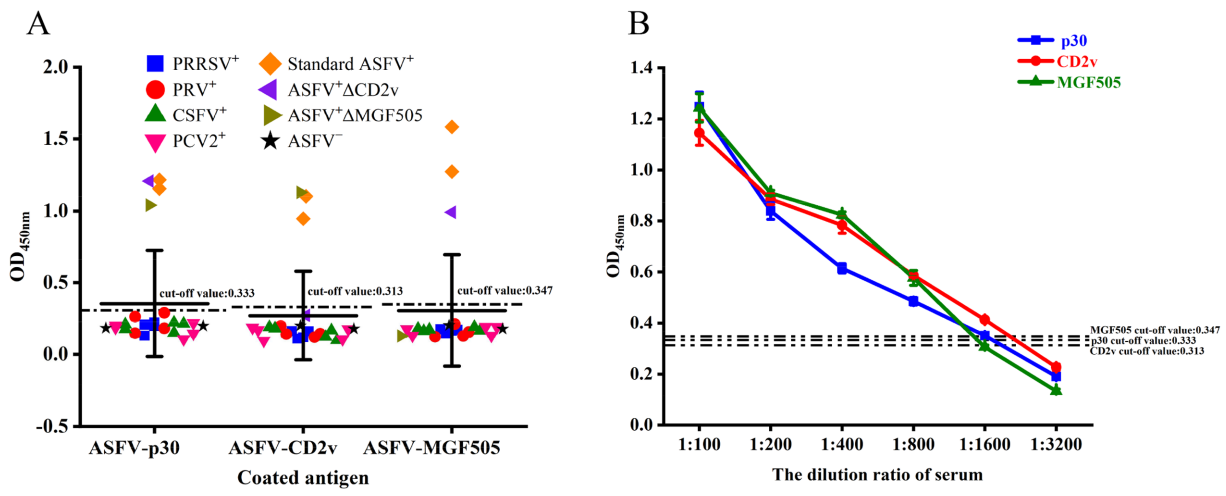

FIGURE 4
Determination of the analytical specificity and analytical sensitivity of the triple protein-based ELISA. The triple protein-based ELISA cannot detect ASFV⁻, PRRSV⁺, PRV⁺, CSFV⁺, and PCV2⁺ serum samples, but can detect standard ASFV⁺, ASFV⁺ΔCD2v, and ASFV⁺ΔMGF505 serum samples at 1:100 dilution **(A)**. Standard serum ASFV⁺ was diluted to test the detection limit of triple protein-based ELISA. According to the cut-off value, the ELISA based on the ASFV-p30 and ASFV-CD2v proteins could detect a 1,600-fold dilution of standard serum ASFV⁺, and the ELISA based on the ASFV-MGF505 protein could detect a 800-fold dilution of standard serum ASFV⁺. The dashed lines indicate the cut-off value **(B)**.

TABLE 3 Repeatability and reproducibility test of the triple protein-based ELISA.

Assay	Serum samples	ASFV-p30		ASFV-CD2v		ASFV-MGF505	
		$\bar{x} \pm SD$	CV%	$\bar{x} \pm SD$	CV%	$\bar{x} \pm SD$	CV%
Intra-assay	S1	1.150 ± 0.039	3.391	1.124 ± 0.026	2.313	1.347 ± 0.041	3.044
	S2	1.247 ± 0.048	3.849	1.220 ± 0.050	4.098	1.244 ± 0.042	3.376
	S3	1.069 ± 0.043	4.022	0.887 ± 0.022	2.480	1.000 ± 0.038	3.800
	S4	1.159 ± 0.056	4.832	1.230 ± 0.040	3.252	0.195 ± 0.009	4.615
	S5	1.130 ± 0.040	3.540	0.915 ± 0.011	1.202	0.933 ± 0.015	1.608
Inter-assay	S1	1.123 ± 0.012	1.069	1.165 ± 0.041	3.519	1.311 ± 0.024	1.831
	S2	1.250 ± 0.059	4.720	1.181 ± 0.055	4.657	1.258 ± 0.022	1.749
	S3	1.109 ± 0.034	3.066	0.927 ± 0.041	4.423	1.089 ± 0.048	4.408
	S4	1.173 ± 0.029	2.472	1.219 ± 0.055	4.512	0.194 ± 0.009	4.639
	S5	1.127 ± 0.043	3.815	0.942 ± 0.034	3.609	0.948 ± 0.022	2.321

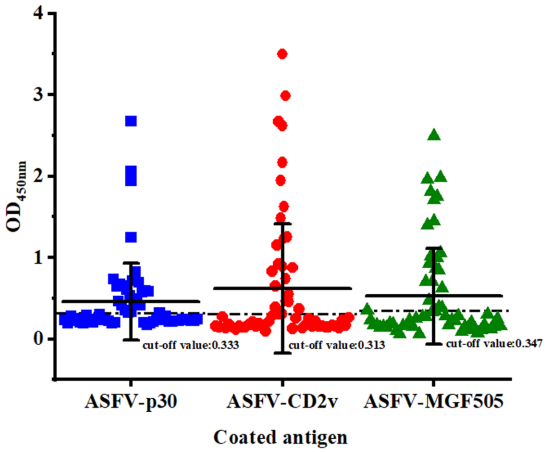

FIGURE 5
Clinical serum samples (n = 59) detection results of the triple protein-based ELISA.

TABLE 4 Comparison of the triple protein-based ELISA with the commercialized triplex qPCR kit and the commercialized ELISA kit.

	The triple protein-based ELISA (+)	The triple protein-based ELISA (−)	Total
The commercialized triplex qPCR kit (+)	22	1	23
The commercialized triplex qPCR kit (−)	0	36	36
Total	22	37	59
The commercialized ELISA kit (+)	22	2	24
The commercialized ELISA kit (−)	0	35	35
Total	22	37	59

the three detection methods are consistent with each other after optimized. Therefore, ASFV-P30, ASFV-CD2V and ASFV-MGF505 proteins can be separately coated on the same microtiter plates and detect the corresponding antibodies simultaneously under the same reaction conditions, which simplified the differential detection process and saved detection time. This newly established triple protein-based ELISA showed good specificity, sensitivity, repeatability and reproducibility. The repeatability and reproducibility exceed those of ASFV ELISA methods established both by Wang et al. (CV value was <20%) and Jiang et al. (CV value was <10%) (17, 42). To evaluate test accuracy and validate whether the newly developed methods could be used to differentiate wild ASFV infection and gene-deleted ASFV infection, a total of 59 clinical serum samples were detected by this triple protein-based ELISA, commercialized triplex qPCR kit based on p72/CD2v/MGF gene (BioKITai, Xiamen, China) and commercialized ASFV antibody detection kit (Luoyang Putai Biological Technology Co., Ltd., China) which use p30 protein as detecting antigen. The results of the triple protein-based ELISA showed that 22 (19 were ASFV$^+$ wild-type, one was ASFV$^+$ΔCD2v, and two were ASFV$^+$ΔMGF505) of the 59 samples were positive for the ASFV antibody. This indicates that wild-type and gene-deleted ASFV strains were simultaneously circulating in northern China. Compared with the detection results of the commercialized triplex qPCR kit (21 ASFV$^+$ wild-type sera, 1 ASFV$^+$ΔCD2v serum, 1 ASFV$^+$ΔMGF505 serum, and 36 ASFV$^-$ sera), the test accuracy was 98.31% (58/59). Compared with the detection results of the commercialized ELISA kit (24 ASFV$^+$ sera and 35 ASFV$^-$ sera), the test accuracy was 96.61% (57/59). These results indicate that the triple protein-based ELISA method not only can effectively discriminate the antibodies induced by wild-type ASFV infection from ASFVΔCD2v virus strain infection but can effectively differentiate among wild-type ASFV, ASFVΔCD2v virus strain, and/or ASFVΔMGFs virus strains infection. When further analysis the detection results of these assays, we found that one of the samples was detected to be ASFV$^+$ΔMGF505 by the triple protein-based ELISA, ASFV$^+$ by the commercial ELISA kit, but ASFV$^-$ by the commercialized triplex qPCR kit. When the PCR detection of the serum sample yields a negative result, the pig either not be infected with ASFV or has infected with lower virulent ASFV and was in the stage of convalescence/virus non-shedding of the disease. The latter is quite likely, since the

positive results of the commercial ELISA and triple ELISA have proved it. Therefore, the triple protein-based ELISA can detect the PCR false negatives due to the postponed or discontinuous virus shedding in naturally gene-deleted strains and can differentiate ASFV^{+}ΔMGF505 from the wild-type. Compared with other commercialized ELISA kits and published ELISA methods, this method is more suitable for the current prevalence of ASFV. It offers an important diagnostic tool for the effective prevention and control of ASFV.

In conclusion, in this study a triple protein-based ELISA method was established based on the proteins ASFV-p30, ASFV-CD2v, and ASFV-MGF505. It was sensitive and specific, and can effectively differentiate the antibodies induced by wild-type ASFV from the gene-deleted ASFV. It will be useful for the serological differential diagnosis and epidemiology study of ASFV. At the same time, the p30, CD2v and MGF505 proteins were considered the key research targets for attenuated and subunit vaccines. Therefore, in the future, this newly developed triple ELISA method can also be used to discriminate vaccine immunization from natural infection. However, because the clinical samples used to evaluate the applicability of the developed ELISA were all collected from suspected ASFV-infected pig farms, therefore, geographical regions and the numbers of samples are limited, more samples from different geographical regions at home and abroad should be collected to further evaluate the newly developed triple protein-based ELISA method to ensure global applicability and consistency before commercialization.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by the Ethics Committee of Hebei Agricultural University, and the approval number is 2021069. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SZ: Data curation, Formal analysis, Methodology, Software, Validation, Writing – original draft, Writing – review & editing. YZu: Conceptualization, Funding acquisition, Investigation, Resources, Writing – review & editing. WG: Resources, Writing – review & editing. YZh: Formal analysis, Software, Validation, Writing – review & editing. YL: Formal analysis, Software, Validation, Writing – review & editing. JF: Conceptualization, Funding acquisition, Investigation, Resources, Writing – review & editing.

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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.

Publisher's note

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Mental health impacts of African swine fever outbreaks on veterinarians in the Philippines

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Emergence of African swine fever (ASF) in the Philippines in 2019 caused substantial impacts on animal health and its pig industry. To control ASF, strict policies were applied including zoning and depopulation of infected herds. While ASF's severe impacts on pigs are well recognized, its potential impacts to public health are often overlooked. ASF is not a food safety concern and does not infect humans, but it has the potential to affect mental, emotional, and social well-being during emergency response. Veterinarians may be particularly at risk due to their role in depopulation of pigs and other distressing tasks. The objective here was to assess the effects of the ASF outbreaks on Filipino veterinarians' mental and social well-being. A questionnaire was created and anonymously administered to 13 Filipino veterinarians attending a training workshop in December 2023. All participants had experience responding to the ASF outbreaks. Then, the summary responses were discussed with the entire group, allowing for clarification and verification. Two-by-two contingency tables and Fisher's exact test were used to explore associations between responses. The top five negative signs reported by >50% participants were "reduced energy," "reduced sleep," "new feelings of hopelessness or sadness," "new feelings of anger or frustration," and "reduced enjoyment of life." Some veterinarians also reported negative social interactions such as antagonism and blame toward public veterinarians. These results highlight the often-overlooked impacts of ASF on mental and social well-being and indicate the need for mental health support for veterinarians as part of comprehensive ASF mitigation efforts.

KEYWORDS

African swine fever, mental health, public health, Philippines, questionnaire, One Health, pigs, social wellbeing

1 Introduction

African swine fever (ASF) is a complex, viral disease caused by the ASF virus (ASFV). ASFV only affects pigs and causes severe hemorrhagic symptoms, fever, and often high mortality nearing 100% of affected pigs (1–4). There is no treatment or vaccine for ASF, and the disease can only be prevented through strict biosecurity of pig farms. Because of its severity and significance to global pig health, ASF is reportable to the World Organisation for Animal Health (5). ASF was first described in Africa in 1921 and spread into Europe in 1957, resulting in the first widescale, global ASF epidemic (6). In 2007, a second wave of global ASF expansion began when the country of Georgia experienced an outbreak of the highly virulent genotype II ASFV strain. The ASFV was suspected of being spread from ASFV-contaminated pork in catering waste from ships that was fed to pigs (7). Since 2007, ASF has displayed transboundary

and transcontinental spread across Europe and Asia, notably being detected in China in 2018 and the Dominican Republic and Haiti in 2021. This spread has made control more difficult and has given ASF global significance through its impacts to animal health and the global economy (3, 8–12).

ASF was detected in the Philippines in July 2019. These outbreaks caused significant disruption, including a 41.7% drop in pig production in 2021 and a 20.5% decrease in registered pigs in March 2023 compared to the same quarter in 2020 (13, 14). Approximately 5 million pigs in the Philippines have been culled due to ASF since 2019 which lead to significant economic impacts on farmers, consumers, and the economic stability of the country (14, 15). The ASF outbreaks continued to expand across the Philippines, to which the Filipino government responded by creating and implementing control programs, such as the National Zoning and Movement (NZM) plan for ASF in 2019 (16). This zoning is broken up into infected, buffer, surveillance, or protected zones. Culling of animals within 1 km from the infected zone and within 5 days or less was also implemented. Given the timing of the epidemic, corresponding with COVID-19 efforts and lack of resources and personnel, this program had varying degrees of success (14). There was pushback from pig stakeholders, however, given that pigs hold important economic value and personal and cultural significance in the Philippines (13). Unfortunately, and despite control efforts, ASF remains present in the Philippines, leading to continuing impacts on pig farmers and pigs, inflation of pork prices, and threatening the food security of vulnerable groups (17).

One Health has been defined as "an approach to address a health threat at the human-animal-environment interface based on collaboration, communication, and coordination across all relevant sectors and disciplines, with the ultimate goal of achieving optimal health outcomes for both people and animals" (18). Using the One Health lens in addressing ASF outbreaks allows for a broad view of the various factors affecting successful ASF control, and the recognition and evaluation of public health aspects of the ASF outbreaks such as food security, mental health, and social well-being. Social well-being refers to relationships and the way individuals interact with each other (19). Mental health refers to an individual's emotional, psychological, and social well-being, and affects how they think, feel, act, relate to others, and handle stress (20). Stress is a physiological and psychological response of the body to situations perceived as demanding or threatening. Biologically, stress activates the sympathetic nervous system, releasing hormones such as cortisol and adrenaline, preparing us to face a challenge. It is a key adaptive mechanism for survival, but when persistent or excessive, it can have detrimental effects on physical and mental health. There are several subtypes of stress, classified according to their duration and the context in which they occur. Work stress is defined as the process by which workplace psychological experiences and demands (stressors) produce both short-term (strains) and long-term changes in mental and physical health and can be the result of an imbalance between job demands and available resources (21). Over time, chronic workplace stress can have negative impacts on an individual's wellness and has been linked to burnout and depression (22).

Public and private veterinarians are key personnel in enacting ASF policies and regulations, such as diagnostic testing for regulatory purposes like movements and surveillance, and overseeing and conducting depopulation of ASF-positive herds. Their critical role may put them in a position to experience adverse mental and social effects from the ASF outbreaks. For example, culling pigs, particularly healthy animals to prevent further disease spread, can cause moral conflict for veterinarians whose role is typically to improve animal health. It could also create antagonism between them and private pig stakeholders. However, these and other potential effects of ASF response on veterinarians' mental and social well-being have not yet been well evaluated or documented in previous works. The objective of this study was to assess the mental health and social well-being of veterinarians responding to the ASF epidemic in the Philippines. This work demonstrates the negative effects of ASF on veterinarians and provides important information for decision-makers that can be used to create support mechanisms for them during ASF response activities.

2 Materials and methods

This protocol was reviewed and approved by the University of Minnesota Institutional Review Board. To assess the effects of ASF on the mental health of Filipino veterinarians responding to ASF, a modified Delphi approach was used whereby individual responses were first anonymously collected, and then reviewed as a group to clarify and enhance interpretation of the findings (23). To gather individual responses, a questionnaire was written and administered in English, an official language of the Philippines, to collect information on mental and social wellbeing of veterinarians responding to ASF in the Philippines and compare between pre- and post-ASF outbreaks (Supplementary File 1). Questions were adapted from the World Health Organization Division of Mental Health Quality of Life assessment (24). A mixture of multiple choice and short answer questions were used. The questionnaire and methods were reviewed and approved by the University of Minnesota Institutional Review Board.

Questionnaire respondents were a convenience sample of veterinarians attending an in-person ASF training workshop at the Agricultural Training Institute-International Training Center for Pig Husbandry (ATI-ITCPH) in Batangas, Philippines, in December 2023. Briefly, these veterinarians were part of a cohort that was selected for a series of ASF training workshops by a joint committee of the Philippine College of Swine Practitioners, the Philippine Department of Agriculture Bureau of Animal Industry, and the ATI-ITCPH. The cohort participants were selected based on their years of experience (1–2 years minimum, 5–10 years preferred) and role in the pig industry, balancing the number of private and public veterinarians invited. This selection process took place in February and March 2023, independent of the study conducted here. The full training cohort ($n = 25$ veterinarians) had a median of 20 years of experience as veterinarians, and all had experience with ASF and had seen the disease in the field. The questionnaire was administered during one of the cohort's training workshops in December 2023. Of the training cohort, 13 veterinarians attended the in-person workshop and therefore were able and eligible to participate in the questionnaire activity. All 13 trainees that were present completed the questionnaire.

The questionnaire was individually and anonymously completed by individuals at the in-person workshop under the facilitation of one of the co-authors (RAS). Prior to completing the questionnaire, the participants reviewed and signed a consent form, in conjunction with the requirements of the University of Minnesota Institutional Review Board. The questionnaire was administered anonymously and online using Qualtrics (25). After the participants completed

the questionnaire, the preliminary aggregated responses were reviewed in an unstructured whole group discussion using the results feature of Qualtrics. Aggregated results were projected to the whole group, in a manner so that individual responses could not be identified as belonging to any particular person. Multiple choice questions were automatically displayed by Qualtrics as bar charts with counts and percentages, and open comment responses were displayed as text. For each question, the participants were invited to freely comment on the results with minimal prompting, including whether they had any confusion with the question, how they felt it did or did not generalize to other veterinarians and pig stakeholders in the Philippines, and anything else they felt was important to discuss. The co-author that was present moderated the discussion and captured notes electronically as the participants discussed each question.

The questionnaire data was analyzed using Statistical Analysis Software version 9.04 (SAS) (26). Frequency tables were produced that summarized the count and percentage for each categorical response. Two-by-two contingency tables were used to explore pairwise associations between the five most frequently mentioned signs. Significance was evaluated using Fisher's exact test, and results were considered significant for p-values less than 0.05. Comments captured during the live whole group discussion were reviewed by the co-authors to further aid in interpretation of the findings.

3 Results

The five most commonly reported negative responses were "reduced energy," "reduced sleep," "new feelings of hopelessness or sadness," "new feelings of anger or frustration," and "reduced enjoyment of life" (Table 1; Supplementary File 2). Over half ($n = 7$) of respondents reported those five negative signs. Amongst the remaining respondents, two individuals reported four negative signs, one individual reported three negative signs, one individual reported two negative signs, one individual reported only one negative sign, and one individual reported no negative signs. Twelve participants (92%) reported that they felt positive about the future. None reported intrusive thoughts about death, dying, or that their family or community would be improved if they were gone, and none reported having started or increased visits to a mental health professional.

Four significant ($p < 0.05$), positive pairwise associations were found between the most commonly reported negative signs (Figure 1). Three of these related to "reduced energy," including "reduced energy" and "reduced sleep"; "reduced energy" and "new feelings of frustration and anger"; and "reduced energy" and "reduced enjoyment of life." Additionally, "reduced sleep" and "reduced enjoyment of life" were significantly and positively associated.

Considering aspects of their social-wellbeing and community involvement, the participants mainly reported increased involvement in the community ($n = 6$, 46%) or no change ($n = 5$, 38%). Only two reported less involvement (15%). The majority ($n = 11$, 85%) indicated that they still treated neighbors the same post-ASF outbreaks, and only three individuals reported that they experienced any negative behaviors from neighbors or social circles. In the comment field, these three reported negative social interactions such as avoiding interactions that they felt would lead to confrontations, and antagonism and blame toward public veterinarians because of farmers' losses to ASF.

During the whole group discussion, the veterinarians described that activities such as depopulation of pig herds, surveillance and reporting cases in the midst of resistant farmers and producers, and imposing livestock movement restrictions and quarantine were stress-inducing events they had experienced. They reported that these results are likely generalizable to other public and private veterinarians involved in the ASF response, but that the mental health impact to pig farmers is likely worse because of the direct impacts of activities like herd depopulation to their livelihoods. They also discussed the limited availability and accessibility of mental health professionals in the Philippines.

TABLE 1 Responses of yes/no mental and social-wellbeing questions from Filipino veterinarians that responded to African swine fever (ASF) outbreaks in the Philippines.

Question	Number of respondents with "Yes" response (%)
Do you feel positive about the future?	12 (92)
Have you experienced reduced energy since the ASF outbreak?	11 (84)
Have you experienced reduced enjoyment of life since the ASF outbreak?	10 (77)
Have you experienced new feelings of anger or frustration since the ASF outbreak?	10 (77)
Have you experienced reduced sleep since the ASF outbreak?	9 (69)
Have you experienced new feelings of hopelessness or sadness since the ASF outbreak?	9 (69)
Do you have trouble concentrating on tasks since the ASF outbreak?	6 (46)
Have you experienced poor memory since the ASF outbreak?	4 (31)
Have you experienced extreme changes in feelings of happiness and sadness since the ASF outbreak?	2 (15)
Do you have less self-worth or less confidence in yourself due to the ASF outbreak?	2 (15)
Have you had any intrusive thoughts about death or dying since the ASF outbreak?	0 (0)
Since the ASF outbreak, have you had any intrusive thoughts that your family or community would be improved if you were gone?	0 (0)
Have you started or increased your visits to a mental health professional since the outbreak?	0 (0)
Have you experienced any negative behaviors from your neighbors or social circle since the outbreak?	3 (23)
Do you still treat your neighbors the same pre-outbreak and post-outbreak?	11 (85)

	Have you experienced reduced energy since the ASF outbreak?	Have you experienced reduced sleep since the ASF outbreak?	Have you experienced new feelings of hopelessness or sadness since the ASF outbreak?	Have you experienced new feelings of anger or frustration since the ASF outbreak?	Have you experienced reduced enjoyment of life since the ASF outbreak?
Have you experienced reduced energy since the ASF outbreak?					
Have you experienced reduced sleep since the ASF outbreak?	9 *p=0.045*				
Have you experienced new feelings of hopelessness or sadness since the ASF outbreak?	7 *p=0.5*	7 *p=0.2*			
Have you experienced new feelings of anger or frustration since the ASF outbreak?	9 *p=0.045*	8 *p=0.1*	7 *p=0.2*		
Have you experienced reduced enjoyment of life since the ASF outbreak?	9 *p=0.045*	9 *p=0.045*	7 *p=0.2*	8 *p=0.1*	

Legend

Number of vets who responded "yes" to both questions
Fisher's exact P-value

Non-significant positive association

Significant positive association

FIGURE 1
Correlation matrix of associations between responses to top reported mental health signs from Filipino veterinarians.

4 Discussion

This study represents one of the first formal assessments of the negative impacts of ASF outbreaks on the mental health and social well-being of veterinarians. According to the veterinarians surveyed here, responding to the outbreaks caused new negative feelings and increased stress. Some participants also reported changes in their social interactions because of antagonism toward their roles as enacting government ASF policies. Generally, veterinarians responding to the outbreaks may experience guilt or fear of retribution. Guilt is closely related to ethics, morality, and personal responsibility and may arise when a person believes they have transgressed a moral principle or failed to fulfill an ethical duty (27, 28). Depopulation of whole herds of animals for disease control can conflict with a veterinarian's desire to promote animal well-being, protect the food supply, and to support farmers, ultimately posing deep ethical and moral dilemmas for the individual (28).

These findings are consistent with reports of mental health impacts from other animal disease outbreaks, such as the foot-and-mouth disease (FMD) outbreaks in 2001 in the UK and in 2010 in Japan (29, 30). Previous studies have shown that, following disease outbreaks, post-traumatic stress disorder symptoms can appear as late as 1 year after the incident (31). Veterinarians in the FMD outbreaks had similar experiences to Filipino veterinary responders in the ASF outbreaks, including being exposed to repeated traumatic experiences such as culling herds (29, 30). Parallels between the FMD crisis and these current results of ASF response in the Philippines include loss of trust in authority, feelings of distress, and changes in social dynamics resulting in poor mental health, among others (29, 30). The experiences that Filipino veterinarians reported in responding to the ASF outbreaks are also consistent with those documented for human healthcare workers in responding to COVID-19. For example, behaviors associated with anxiety, depression, and sleep disturbances were similar between these groups (31). The changes in social behaviors reported by some of the respondents also parallel those shown by first responders in COVID-19 pandemic such as experiencing stigma, isolation, and social rejection (31, 32). Despite ASF not being a zoonotic epidemic, which would imply a greater fear of contagion for oneself or one's loved ones, the consequences can be equally devastating for the health of veterinarians (33).

Significant associations between reduced energy, reduced sleep, new feelings of frustration and anger, and reduced enjoyment of life were also observed here. Sleep loss is closely related to emotional conditions such as anxiety and can disrupt emotional and cognitive functioning (34, 35). For veterinarians in emergency response, such as for ASF, increased time working and potential distress from tasks like animal depopulation may disrupt their regular sleeping patterns. Total or partial sleep deprivation has been significantly associated with increased anxiety levels (36, 37).

Together, and if not managed, the negative interdependence of anxiety and lack of sleep can lead to significant emotional and cognitive consequences including increased vulnerability to depression and suicidality (35, 38, 39), emphasizing the need for effective management of both during emergency response.

The current study aimed to primarily document negative impacts, and therefore, potential positive impacts or experiences were not thoroughly captured or explored here. Despite reporting numerous negative feelings and increased stress, nearly all the participants responded that they still felt positive about the future. Arguably, it is possible that for some individuals, and under certain circumstances, there could be positive experiences associated with the ASF outbreaks. For example, in the 2001 FMD outbreaks in the UK, affected individuals reported that formal and informal support networks were a source of strength and helped to alleviate some suffering. In other disease and non-disease disasters and traumatic events, similar positive experiences have been reported, such as community closeness, increased resilience, and personal growth (40–42). However, these studies also highlight the complexity of individual responses to traumatic events. More work is needed to understand the variety and intensity of emotional and behavioral responses from exposure to animal health emergencies such as ASF.

This study had some important limitations and considerations. The surveyed population represented a convenience sample and the overall sample size was small, so these responses may not capture the wide range of experiences and viewpoints of Filipino veterinarians responding to ASF. Using a mainly closed-ended questionnaire provided a standardized way to collect individual responses within a limited timeframe (i.e., during a training workshop), but did not allow for participants to give open responses, which can limit the types of experiences captured. Interactive methods, such as interviews and focus groups, can be conducted in semi-structured ways with open-ended questions and with flexibility for follow-up questions between the researcher and participants (43). This can allow for a deeper exploration of participants' expertise and experiences. Future research should utilize these techniques to capture a wide range of experiences during the ASF outbreaks, ultimately achieving greater qualitative data saturation and exploring the potential complexity that may be present (44). Additionally, the COVID-19 pandemic was occurring at the same time as the initial ASF outbreaks in the Philippines, and its effects may have exacerbated and/or masked the effects observed here from the ASF outbreaks. This study did not examine the effects on pig farmers, which require additional consideration and research. Pig farmers may face their own unique challenges to their mental and social well-being that would require additional support.

The ongoing ASF outbreaks in the Philippines have caused significant impacts on the country. Here, the negative impacts of ASF on veterinarians' mental and social well-being were demonstrated, including reduced energy, reduced sleep, new feelings of anger and frustration, new feelings of hopelessness and sadness, and reduced enjoyment of life. These findings highlight the need to provide mental health training and preparedness for ASF responders to prevent and reduce negative mental and social impacts. Further work will help researchers and policymakers to understand the full vulnerabilities of at-risk communities such as veterinarians and allow for the creation of appropriate support mechanisms. Future studies should aim to further characterize ASF's impacts on physical, mental, social, and economic wellbeing. Expanding this work to other ASF-affected countries will strengthen the ability to fully and accurately assess the public health impacts of an ASF outbreak and develop holistic mitigation strategies.

Data availability statement

The datasets presented in this article are not readily available because restrictions apply to the datasets: the datasets presented in this article are not readily available because of privacy and sensitivity concerns. Requests to access the datasets should be directed to Rachel Schambow, scham083@umn.edu.

Ethics statement

The studies involving humans were approved by University of Minnesota Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

HB: Formal analysis, Writing – review & editing, Writing – original draft. AlP: Writing – review & editing. RM-S: Writing – review & editing. AnP: Conceptualization, Writing – review & editing. RS: Formal analysis, Conceptualization, Writing – review & editing.

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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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1519270/full#supplementary-material

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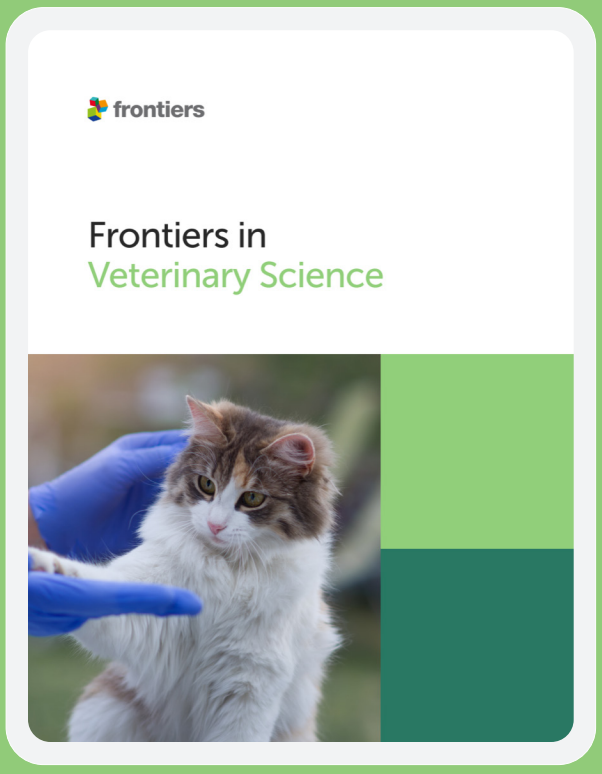

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