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ORIGINAL RESEARCH ARTICLE

Synthetic p72 Peptide-Based Indirect- Enzyme-Linked Immunosorbent Assay for Diagnosis of African Swine Fever Virus Infection

Navdeep Kaur Dhiman[1] · M. Manu[1] · Vinay G. Joshi[2] · Yashpal Singh Malik[1,3]

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Abstract In the present study, we explored the potential diagnostic application of B cell linear peptides derived from the p72 protein of African Swine Fever Virus (ASFV) using a two-step bioinformatics approach for developing an indirect enzyme-linked immunosorbent assay (ELISA) for ASF detection. Through computational analysis, eight linear B cell epitopes with significant conservation across various ASFV genotypes were identified. These peptides were chemically synthesized and evaluated for their immunoreactivity using specific rabbit hyperimmune serum against the ASFV p72 protein. The synthesized peptides displayed notable reactivity in dot-ELISA and subsequently in indirect-ELISA. Validation of the indirect ELISA was conducted using samples collected during ASFV outbreaks in the northern states of India from 2022 to 2023, and further corroborated using a commercial kit. The P1 peptide-based indirect ELISA demonstrated a sensitivity of 100% and a specificity of 86%. Additionally, peptides P3, P4, P5, P7, and P8 exhibited a specificity of 100%, while peptides P2, P3, P4, P6, and P7 displayed a sensitivity greater than 70% in the indirect ELISA. These results underscore the diagnostic potential of p72 protein-specific B cell epitopes for the detection of ASFV infection in field settings. The developed assay holds promise for ASFV infection detection, as well as for seromonitoring and serosurveillance applications.

Keywords African swine fever virus (ASFV) · Synthetic peptides · Immunoinformatic · B-cell epitopes · ELISA · Diagnosis

M. Manu and Vinay G. Joshi have contributed equally to this work.

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✉ Yashpal Singh Malik
malikyps@gmail.com

Navdeep Kaur Dhiman
ndeep.dhiman001@gmail.com

M. Manu
manumohankrishna123@gmail.com

Vinay G. Joshi
vinaygjoshi18@luvas.edu.in

[1] College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141004, India

[2] Department of Animal Biotechnology, Lala Lajpat Rai University of Animal and Veterinary Sciences, Hisar, Haryana 125001, India

[3] Mukteswar Campus, ICAR-Indian Veterinary Research Institute, Mukteswar, Nainital, Uttarakhand 263138, India

Introduction

African swine fever (ASF), a highly contagious viral disease of pigs is caused by the African swine fever Virus (ASFV), the only member of the *Asfarviridae* family. ASFV is a large, double-stranded DNA virus with a complex genome that encodes for over 150 proteins including structural and non-structural proteins. ASFV carries a 170–190 Kbp linear, double-stranded DNA genome encoding 68 structural and 150–200 non-structural proteins [1]. It has an icosahedral structure: an outer envelope, capsid, inner membrane, core shell, and genome [2].

ASF was initially identified in Kenya back in 1921 [3]. The virus was maintained within a sylvatic cycle involving African wild pigs, warthogs, and *Ornithodoros* soft tick vectors, but it also eventually infiltrated domestic pig

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populations in the region [4]. Europe encountered first ASFV outbreak during the 1950s. ASFV was reintroduced to Europe via infected wild boars in Georgia, leading to its spread across Russia, Ukraine and neighboring nations [5]. The disease was first introduced in the north-eastern region of India in 2020, subsequently spreading to all regions of the country and emerging as a significant threat to the pig population. ASFV has a significant ability to replicate in macrophages, influencing their function and evading host immune defenses [6–8]. The outer capsid protein p72, encoded by the *B646L* gene, serves as the primary component, comprising 33% of the viral particle's composition [9]. The p72 protein plays a crucial role in the assembly of viral capsids and contributes to the process of virus adsorption and invasion to susceptible cells [2]. Its dual characteristics, conserved and immunogenic, makes it a pivotal antigen in various diagnostic method advancements [9, 10]. Though, various serological assays are available but their sensitivity sometime is compromised [11]. To combat this, synthetic peptide infused immunoassay comes as a rescue.

The advancements in the field of bioinformatics have had a transformative impact on the development of peptide-based (epitope) vaccines and diagnostics. Several methods for diagnosis, such as ELISA (Enzyme-Linked Immunosorbent Assay), lateral flow devices, and microarrays, have been developed to detect viral diseases using peptides. Synthetic peptides can be designed to identify chemically defined antigen as an alternative to natural protein antigen. They can also be tailored to target specific regions of viruses or proteins making them potentially more precise. The p72 is a significant capsid protein of ASFV and plays a crucial role in determining the genotypes. ASFVs are classified into 24 genotypes according to the C-terminal sequence of their B646L gene(p72), with a nucleotide identity ranging from 86.2% to 99.5%. The p72 protein is widely recognised as the primary immunogenic protein, making it an ideal candidate for the development of serodiagnostic tools. Researchers utilised prokaryotic and eukaryotic expressed p72 protein to develop several monoclonal antibodies. These antibodies were then investigated for their potential in the advancement of diagnostic tools [12, 13]. Researchers have developed various forms of ELISA assays to specifically detect ASFV antibodies by utilising the p72 protein [14, 15]. Multiple studies have shown that the immunochromatographic assay based on p72 protein exhibits a high level of sensitivity and specificity [15–18]. Because of its conservative nature and immunogenic properties, it is extensively employed as an antigen in the creation of diagnostic techniques for all genotypes of ASFV. Currently, there is a lack of diagnostic assay kits that are specifically designed for the detection of antibodies suitable for prevalence studies. The current investigation identified eight distinct peptides derived from the p72 protein of ASFV and subsequently developed an indirect ELISA for the precise detection of antibodies against ASFV. The ELISA that was developed has been validated using a commercial ELISA kit (AsurDx™ African Swine Fever Antibody Test, BioStone Animal Health). The developed indirect ELISA demonstrated high sensitivity and specificity in detecting antibodies against ASFV, thus offering a novel surveillance tool for ASFV monitoring.

Materials and Methods

Amino Acid Sequence of p72 Protein

The nucleotide sequence of p72 protein for genotype II was obtained from GenBank (Accession Number ON875961.1). For conservancy analysis, genotypes I (MN886939.1), II (ON 875961.1), X (AY578698.1) and XXIII (KT795354.1) were chosen due to the availability of their complete coding sequences. This selection enabled an examination of the conservation levels within the p72 protein across genotypes I, X and XXIII.

Prediction of B-Cell Epitopes

The determination of B-cell epitopes in this study involved the utilization of the BepiPred 2.0 tool, which employs the random forest regression (RF) algorithm, by inputting the sequence. This algorithm is trained on epitopic data derived from crystal structure. The employed methodology utilized a five-fold cross-validation approach, incorporating hydrophobicity, polarity, surface accessibility, secondary structure, and computed values. Only peptides with an amino acid range exceeding 10 amino acids were chosen for selection in this study.

IEDB Analysis

The Immune Epitope Database and Analysis resource utilizing pre-determined amino acid and antigen sequence characteristics was applied to identify the p72 protein linear B-cell epitopes of ASFV. The analysis involved the evaluation of flexibility, surface accessibility, and hydrophilicity predictions through the application of three separate algorithms: Karplus-Schulz flexibility (with a threshold of 0.9), Emini surface prediction (with a threshold of 1.0), and Parker hydrophilicity prediction (with a threshold of 1.62). The peptides were chosen based on their area under the curve (AUC) characteristics during the selection of overlapping sequences.

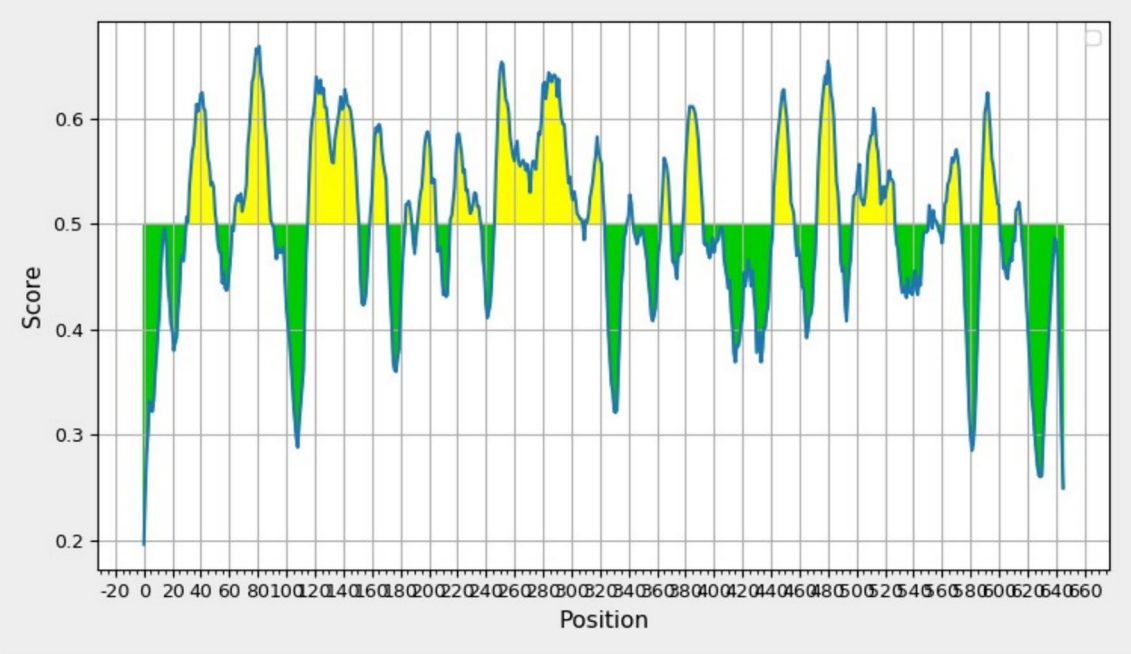


Fig. 1 The results of Bepipred-2, hosted by the online server IEDB, predicted linear B-cell epitopes and the determined threshold value was 0.5

Table 1 The list of predicted peptides retrieved from BepiPred 2.0 tool

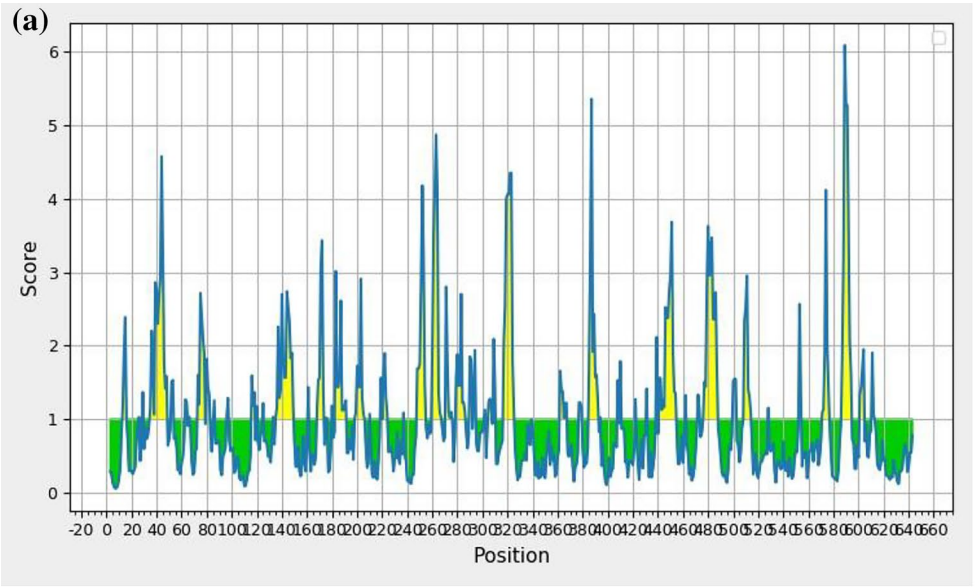
S. No.	Start	End	Peptide	Length
P1	31	52	SNIKNVNKSYGKPDPEPTLSQI	22
P2	65	91	KPYVPVGFEYNKVRPHTGTPTLGNKLT	27
P3	116	172	SWQDAPIQGTSQMGAHGQLQTFPRNGYDWDNQ TPLE	36
P4	160	172	PFGRPIVPGTKNA	13
P5	193	206	FDVNGNSLDEYSSD	14
P6	216	237	IPGDKMTGYKHLVGQEVSVEGT	22
P7	247	308	DLHKPHQSKPILTDENDTQRTCSHTNPKFLSQHF PENSH-NIQTAGKQDITPITDATYLDIRR	62
P8	311	324	HYSCNGPQTPKYYQ	14
P9	380	393	RFIAGRPSRRNIRF	14
P10	443	457	VTHTNNNHHDEKLMS	15
P11	473	489	TWNISDQNPHQHRDWHK	17
P12	498	528	MQPTHHAEISFQDRDTALPDACSSISDISPV	31
P13	563	574	PFHYGGNAIKTP	12
P14	589	601	REEYQPSGHINVS	13

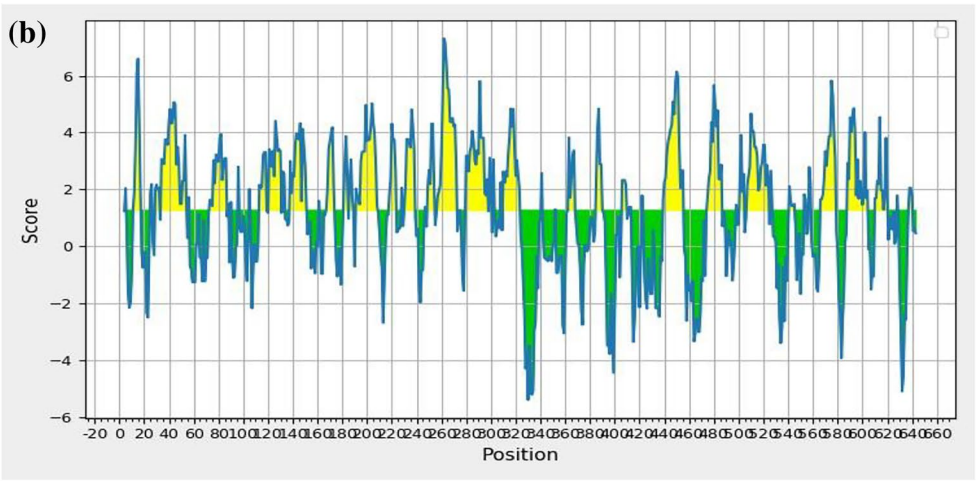
ABCPred and BCEPred Prediction

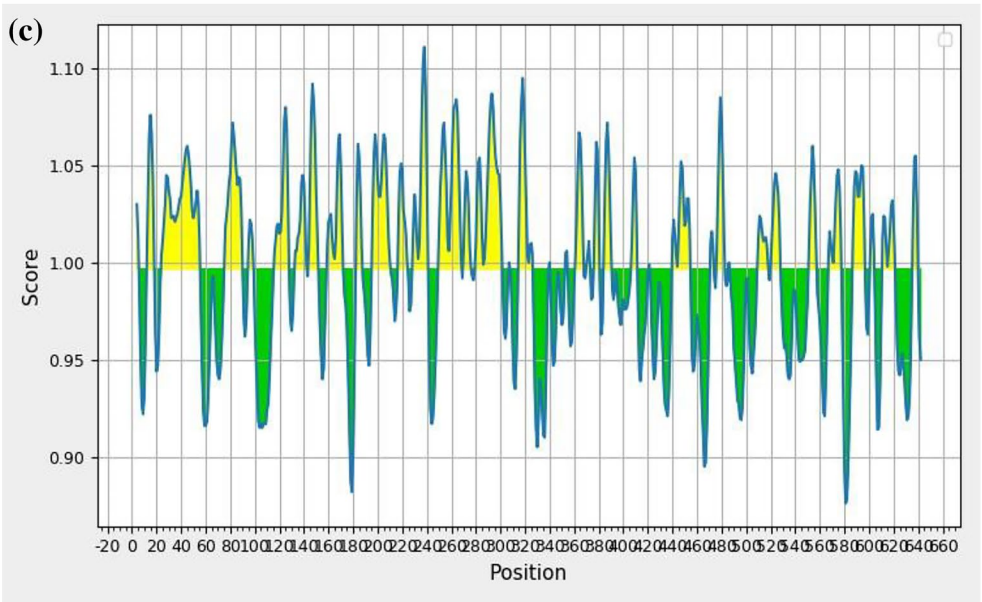
In order to improve the precision of predictions, machine learning tools were used. The present analysis relies on two distinct prediction methods, namely BCEPred, an algorithm that uses Support Vector Machines and ABCPred, which is based on Artificial Neural Networks.

Protean 3D Analysis

The Protean 3D tool employs a set of algorithms in order to visually represent the secondary structure of proteins. The algorithms used in this study included the Garnier-Osguthorpe-Robson (GOR) algorithm, the Chou-Fashman Algorithm, the Kytee Dolittle algorithm for hydrophilicity, the Eisenberg plot methodology for amphipathic regions, and the Jameson Wolff for antigenic index. The Protean was


(a)
Score
Position


(b)
Score
Position


(c)
Score
Position

◂**Fig. 2** **a** The results of the Emini Surface Prediction tool where yellow-coloured regions exceed the specified threshold and are predicted to constitute a part of the linear B cell epitope. Conversely, the green regions are non-antigenic. The threshold value is set at 1.0. In this representation, the Y-axis represents residue scores, while the X-axis corresponds to the positions of residues within the sequence. **b** Result of Karplus-Schulz Flexibility where regions that are yellow-coloured exceed the specified threshold and are predicted to constitute a component of the B cell epitope. Conversely, the green-coloured regions are considered non-antigenic. The threshold value is set at 0.9. In this context, the Y-axis represents residue scores, while the X-axis represents the positions of residues within the sequence. **c** Result of Parker Hydrophilicity Prediction. The regions that exceed the designated threshold are hypothesized to constitute a component of the B cell epitope. Conversely, the regions highlighted in green are deemed non-antigenic, with a threshold value of 1.62. In this context, the Y-axis represents residue scores, while the X-axis represents the positions of residues within the sequence (color figure online)

also used to analyse the physio-chemical properties of the selected peptides. The service of this technique facilitated a thorough investigation of the entire sequence, thereby enabling a more precise analysis of the epitopes. This enabled a more precise delineation of the chosen sequence.

Conservancy Analysis

The amino acid sequences coding the p72 protein from various genotypes, encompassing complete coding sequences, were retrieved from the NCBI database. Subsequently, sequences were analysed in relation to the eight discrete peptides that had been identified in the present study. This tool facilitated the acquisition of knowledge regarding the structural characteristics of proteins, specifically the identification of α-helices, β-sheets, and coils. Significantly, the algorithms underwent training using datasets that consisted of both epitopes and non-epitopes. The analysis facilitated the identification of similarities among different genotypes. The identity was set to a maximum of 100% to developing a widely accepted diagnostic approach that encompasses the primary genotypes of ASFV is advantageous.

Polyclonal Sera and Serum Samples

Polyclonal rabbit serum raised against p72 protein of the ASFV available in the Molecular Diagnostic and Vaccinology Laboratory, College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, was used. The serum samples, both positive and negative, obtained during the ongoing ASFV outbreaks that occurred in the northern states of India during 2022–23, available in the laboratory were utilised in the present study for validating the results.

Designing of Peptides

By employing computational tools, we successfully identified eight linear B cell epitopes that exhibit a significant level of conservation across multiple genotypes of ASFV. The selected peptides were commercially synthesised.

Dot-ELISA and Indirect ELISA to Check the Reactivity of Peptides by Using Polyclonal Sera Raised in Rabbits Against p72 Protein

(i) Dot-ELISA

In the dot-ELISA, a 5 µl drop of peptide solution with a concentration of 200 ng/µl was applied onto a nitrocellulose membrane and subsequently dried for a duration of 5 min at a temperature of 37 ℃. It was thereafter subjected to three rounds of washing, with each round lasting for a duration of 2 min, using a 0.1% solution of PBS-T (Phosphate-buffered saline with Tween-20). Following this, the membrane was subjected to a blocking procedure at a temperature of 37 ℃ for a duration of 1.5 h by using a solution consisting of 5% Skim milk powder (SMP) + 5% Bovine serum albumin (BSA) in 0.05% PSB-T. Following that, the membrane underwent three additional washes. The primary antibody, raised in rabbits against the p72 protein of ASFV and in a 1:20 dilution in the blocking buffer, was added and allowed to incubate for 45 min at a temperature of 37 ℃. Following three washes, a secondary antibody, specifically anti-rabbit horseradish peroxidase conjugated (anti-rabbit HRPO), was used at a dilution of 1:2000. The incubation period lasted for 40 min at a temperature of 37 ºC. After performing three more washes, colour development was initiated using 6 mg of 3,3'-diaminobenzidine (DAB) dissolved in 10 ml of PBS, along with the addition of 10 µl of hydrogen peroxide (H_2O_2). The reaction was terminated by the addition of an excess amount of double-distilled water.

(ii) Indirect ELISA

Ninety-six-well ELISA plates were coated with synthetic peptides (100 ng/well) in Citrate Phosphate Buffer (CPB) overnight at 4 °C. Following, a 2-h incubation at 37 °C in a 10% blocking solution (composed of 5% SMP and 5% BSA) in 0.05% PBS-T, the plates were subjected to three washes in 0.05% phosphate-buffered saline with Tween-20 (PBS-T). Subsequently, the plates were exposed to a 1:100 dilution of rabbit hyperimmune sera against p72 protein of ASFV at 37 °C for a duration of 45 min. After performing three washes with 0.1%PBST, a secondary antibody against rabbit IgG conjugated with horseradish peroxidase (HRPO)

Table 2 The peptides after IEDB analysis using various indices along with overlapped sequences obtained with BepiPred 2.0 tool

S. No.	Start	End	Peptide	Length
P1	31	52	SNIKNVNKSYGKPDPEPTLSQI	22
P2	116	172	SWQDAPIQGTSQMGAH-GQLQTFPRNG YDWDNQTPLE	36
P3	216	237	IPGDKMTGYKHLVGQEVSVEGT	22
P4	311	324	HYSCNGPQTPKYYQ	14
P5	441	455	TQVTHTNNNHHDEKL	15
P6	477	487	DQNPHQHRDW	10
P7	571	576	IKTPDD	6

was applied at a dilution of 1:6000 for a duration of 40 min at a temperature of 37 °C. Following six washes with 0.1% PBST the 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was introduced to induce colour development. The reaction was subsequently terminated by the addition of 2 M sulfuric acid (H_2SO_4), and the optical density (OD) values were determined at a wavelength of 450 nm.

Procedure of Peptide Based Indirect ELISA

Ninety-six-well ELISA plates were used for coating each peptide in citrate phosphate buffer. Each well was coated with different quantities of peptides (100, 250, 500 and 1000 ng) and subsequently incubated overnight at a temperature of 4 °C. On the subsequent day, the unbound peptide was disposed of without undergoing any washing procedure. The blocking process involved incubating the sample at a temperature of 37 °C for a duration of 2 h. A total volume of 300 µl of 10% blocking solutions (composed of 5% SMP and 5% BSA in 0.05% PBS-T, 5% SMP alone in 0.05% PBS-T, 5% BSA alone in 0.05% PBS-T and 5% Foetal bovine serum) were used for standardization of indirect ELISA. The blocking solution was prepared in 0.05% phosphate-buffered saline with Tween-20 (PBS-T). Following the blocking step, the plates were subjected to three consecutive washes using a 0.1% solution of PBS-T. The known positive and negative pig sera samples were used for standardization at different dilutions (twofold dilutions) and incubated for 45 min at a temperature of 37 °C in a blocking solution. Subsequently, the sample was subjected to three additional washes using a solution of 0.1% PBS-T. Subsequently, the plates were subjected to incubation with 100 µl of secondary antibody (anti-porcine antibodies horseradish peroxidase conjugated HRPO) at different dilutions (1:1000 to 1:10,000) per well and maintained at a temperature of 37 °C for 40 min. Following incubation with the secondary antibody, the plate washed six times with 0.1% PBS-T. To initiate colour development, 50 µl of TMB substrate solution containing H_2O_2 was added to each well. The reaction was halted by the addition of 2 M sulfuric acid (H_2SO_4). The measurement of optical density (OD) was performed at a specific wavelength of 450 nm and the obtained values were adjusted by subtracting the optical density of the blank control.

Fig. 3 The peptides that were chosen for further investigation were identified through the application of ABCPred and BCEPred analyses. Among these peptides, the ones exhibiting the highest degree of sequence overlap with the tools were highlighted in red and subsequently selected for further study (color figure online)

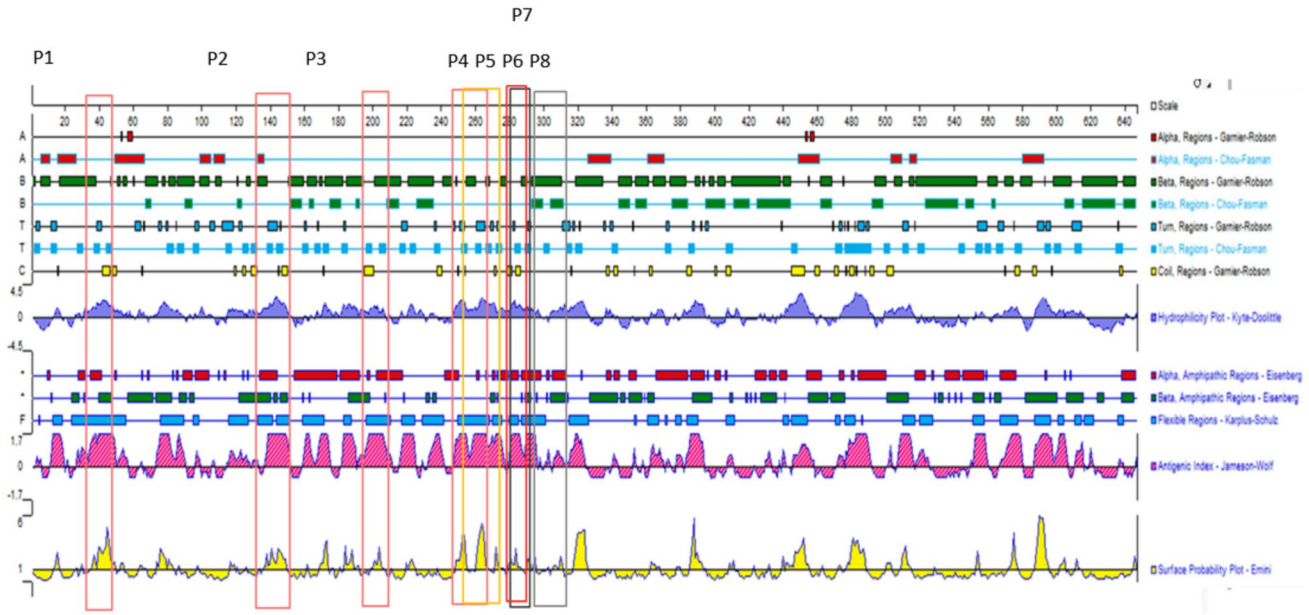


Fig. 4 Protean 3D-DNAStar Analysis: The peptides within the highlighted region represent the subset of sequences that underwent comprehensive filtering using multiple tools

Table 3 Finalized Peptides: Derived from analysing the most overlapping sequences in sequence-based tools and machine learning algorithms

S. No.	Peptide sequence	Peptide length
P1	VNKSYGKPDPEPTLSQ	16 amino acids
P2	TFPRNGYDWDNQTPLE	16 amino acids
P3	VNGNSLDEYSSDVTTLV	17 amino acids
P4	DLHKPHQSKPILTDENDT	18 amino acids
P5	PILTDENDTQRTCSHTN	17 amino acids
P6	HFPENSHNIQTAGK	14 amino acids
P7	FPENSHNIQTAGKQD	15 amino acids
P8	ITPITDATYLDIRRNV	16 amino acids

Field Samples Screening

The serum samples (n = 17), collected during ASF outbreaks in northern states (2022–2023), available in the laboratory were tested with commercial ASFV antibody detection kit (AsurDx™ African Swine Fever Antibody ELISA Test Kit, BioStone Animal Health). Based on the results of the commercial kit, the samples were categorised as either negative or positive for ASF. The positive and negative serum samples were used to evaluate and develop indirect ELISA. The cross-reactivity of the indirect ELISAs that were developed was evaluated using serum samples that tested positive for Classical swine fever.

Statistical Analysis

The present study employed statistical analysis to scrutinize the data and extract meaningful insights. Statistical analyses were conducted using GraphPad Prism 10.0 software. Utilizing this software, the receiver operating characteristic (ROC) curve was generated based on the positive and negative serum optical density (OD) values. This facilitated the determination of the cut-off point, sensitivity, and specificity of the assay. The Kappa values for the developed peptide ELISAs were calculated.

Results

B-Cell Epitope Prediction

The identification of peptides was facilitated using BepiPred 2.0 with a threshold of 0.5, as illustrated in Fig. 1. yellow region represents the presence of antigenic peptide region with a higher degree of antigenicity, whereas the green region corresponds to peptides that have a lower tendency to induce an immune response. The Y-axis corresponds to the scores of residues, while the X-axis corresponds to the positions of residues in the sequence. This analysis resulted in the identification of 14 unique antigenic peptide regions, each spanning more than 10 amino acids (Table 1). Additionally, the Immune Epitope Database (IEDB) analysis

Table 4 Conservancy analysis used to check the epitope conservancy among different common genotypes (I, X, and XXIII). The maximum identity for the output was set to 100%

Epitope	Epitope sequence	Sequence matches at < = 100%	Minimum identity (%)	Maximum identity (%)	Matched genotypes	Non-Matched genotypes
P1	VNKSYGKPDPEPTLSQ	100.00% (3/3)	100.00	100.00	I, X, XXIII	–
P2	TFPRNGYDWDNQTPLE	100.00% (3/3)	100.00	100.00	I, X, XXIII	–
P3	VNGNSLDEYSSDVTTLV	66.67% (2/3)	94.12	100.00	I, X	XXIII (16th aa A rather V)
P4	DLHKPHQSKPILTDENDT	33.33% (1/3)	88.89	100.00	I	X (M rather L) XXIII (M, M rather than L, K)
P5	PILTDENDTQRTCSHTN	33.33% (1/3)	94.12	100.00	I	X, XXIII (14th aa T rather S)
P6	HFPENSHNIQTAGK	100.00% (3/3)	100.00	100.00	I, X, XXIII	–
P7	FPENSHNIQTAGKQD	100.00% (3/3)	100.00	100.00	I, X, XXIII	–
P8	ITPITDATYLDIRRNV	100.00% (3/3)	100.00	100.00	I, X, XXIII	–

aa- amino acid

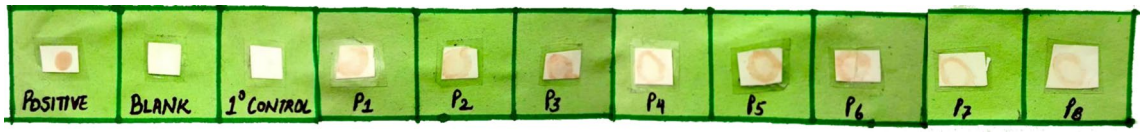


Fig. 5 Dot- ELISA (on Nitro-Cellulose Membrane): Positive: p72 purified protein; Blank: Antigen control; 1° Control: No Hyper immune sera; P1–P8: Peptides reactivity with hyperimmune p72 specific rabbit sera

Table 5 The details of sample screening using a commercial ELISA kit and indirect ELISAs with peptide 1 to peptide 8

Sample ID	Commercial ELISA	P1 ELISA	P2 ELISA	P3 ELISA	P4 ELISA	P5 ELISA	P6 ELISA	P7 ELISA	P8 ELISA
N1	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative	Negative
N2	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
N3	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
N4	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
N5	Negative	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
N6	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
N7	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Negative
P1	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
P2	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
P3	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
P4	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
P5	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
P6	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Positive	Negative
P7	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative
P8	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
P9	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative
P10	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative	Negative

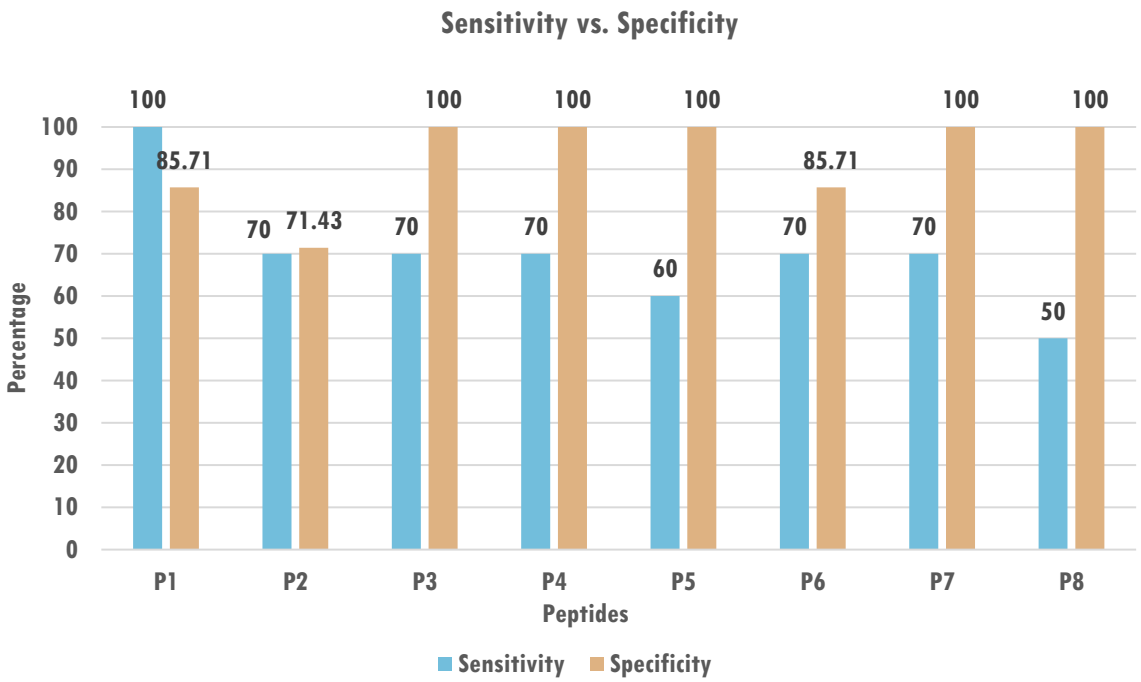


Fig. 6 The plot shows sensitivity and specificity of the developed ELISA were assessed by analyzing the optical density values of positive and negative samples using ROC curve analysis in Prism software (the details of ROC curve analysis included in the supplementary file)

Table 6 The ELISA results for multiple peptides (P1 to P8) and their respective calculated kappa values

Serial No	ELISA	Positive	Negative	Sensitivity (%)	Specificity (%)	Kappa value
1	P1 ELISA	10/10	6/7	100	85.71	0.8759
2	P2 ELISA	7/10	5/7	70	71.4	0.4056
3	P3 ELISA	7/10	7/7	70	100	0.6577
4	P4 ELISA	7/10	7/7	70	100	0.6577
5	P5 ELISA	6/10	7/7	60	100	0.5526
6	P6 ELISA	7/10	6/7	70	85.71	0.5342
7	P7 ELISA	7/10	7/7	70	100	0.6577
8	P8 ELISA	5/10	7/7	50	100	0.4516

using various indices revealed the likelihood of seven additional antigenic peptides, as depicted in Fig. 2a, b, and c. This finding was made through comparative assessment with the outcomes obtained from BepiPred 2.0 (Table 2). The peptide regions identified by BepiPred were further critically analyzed using ABCPred and BCEPred tools, with the results presented in Fig. 3 to align the findings. The enhanced prediction methodology facilitated the selection of specific peptides with better prospects from Table 2. Notably, peptides P4, P5, P6, and P7 were excluded from the analysis, with P7 being excluded specifically due to its short length. A comparative analysis was conducted using Protean 3D (Fig. 4). The findings, presented in Table 3 and depicted in the accompanying figure, led to the discovery of a total of eight peptides. These peptides consist of amino acid sequences ranging from 14 to 18 residues in length. Subsequently, the sequences underwent a conservancy analysis, as outlined in Table 4, involving genotypes I, X, and XXIII. Peptides sequences P1, P2, P6, P7, and P8 exhibited no changes in genotypes I, X, XXIII, and II of ASFV, with identity ranging from a minimum of 88.89% to a maximum of 100%. The study suggests that the identified peptides can be utilized in the development of diagnostics for various genotypes of ASFV as well.

Enzyme Linked Immunoassay

i. Peptide Dot-ELISA

The Dot-ELISA analysis of the 8 designed peptides revealed significant activity against the hyperimmune serum raised against the p72 protein of ASFV (Fig. 5).

Peptides P1, P2, P3, P5, and P6 displayed the highest level of reactivity with the hyperimmune serum, while P4, P7, and P8 exhibited a moderate level of reactivity. Similarly, the indirect ELISA analysis indicated notable activity of all peptides against the hyperimmune serum generated against the p72 protein of ASFV. Peptides P1, P3, P5, P6, and P7 showed the most pronounced reactivity, with optical density values exceeding 1.0. Conversely, P2, P4, and P8 demonstrated a moderate level of reactivity, with optical density values above 0.7.

ii. Indirect Peptide ELISA

During the standardization of peptide ELISA, it was found that using 250 ng of each peptide for coating and overnight incubation at 4 °C yielded the most favorable outcomes in terms of optical density values. The blocking solution composed of 5% SMP and 5% BSA in 0.05% PBS-T was identified as the optimal blocking solution for the peptide ELISA. Diluting the serum at a ratio of 1:100 resulted in optimal results. Additionally, the anti-pig HRPO conjugate at a dilution of 1:6000 demonstrated excellent performance. Peptides P1, P3, P4, and P7 exhibited the highest positive-to-negative optical density (OD) ratio in the developed ELISA. These standardized conditions were utilized for sample testing and validation of the developed indirect ELISA.

Sample Testing

Seventeen field samples collected during outbreaks were initially screened using a commercial ELISA kit, resulting in ten samples testing positive and seven negatives for ASFV-specific antibodies. These samples were then employed to validate the peptide-based indirect-ELISA. Each sample underwent testing using all eight selected peptides, with the results (Table 5) and their corresponding Area Under the Curve (AUC) values presented in the figures (Supplementary file). Peptide P1 demonstrated a sensitivity of 100% and a specificity of 86%. Peptides P3, P4, P5, P7, and P8 demonstrated a specificity of 100% in the indirect ELISA. Peptides P2, P3, P4, P6, and P7 exhibited sensitivities greater than 70%, while P5 and P8 had sensitivities of 60% and 50% respectively. (Supplementary File). The plot depicting the sensitivity and specificity of ELISA based on different peptides is illustrated in Fig. 6. The Kappa coefficient for the peptide ELISA with peptide 1 was 0.87, indicating excellent agreement. The remaining three peptides (P3, P4, and P7) had Kappa values above 0.65, which are considered substantial. The remaining four peptide ELISA had kappa values above 0.4, indicating moderate values (Table 6).

The peptide ELISAs had been evaluated for cross-reactivity using serum samples that were positive for the Classical Swine Fever Virus (CSFV). Classical swine fever is an important pig disease. It is often challenging to differentiate between CSFV and the African Swine Fever Virus. In this study, we attempted to determine the cross-reactivity of CSFV by testing it with serum samples from pigs that tested positive for CSFV. The CSFV positive samples tested negative in all eight peptide ELISA assays, indicating a lack of cross-reactivity with CSFV antibodies.

Discussions

The ASF disease poses a significant and widespread threat to global pig populations. The presence of diverse virus genotypes and the absence of an effective vaccine underscore the urgent need for rapid detection methods. While PCR and isothermal amplification techniques offer high precision, they entail considerable costs and are susceptible to carry-over contamination, potentially leading to erroneous positive results in field conditions. Serological approaches, such as ELISA, present viable alternatives; however, the accuracy of these methods may be compromised by sample handling procedures. Over the past decade, there has been notable progress in the development of peptide ELISA assays tailored for highly sensitive and specific detection of antibodies against various animal viruses. Customizable synthetic peptide-based immunoassays have emerged as promising diagnostic tools, as evidenced by successful applications in other porcine viruses such as rotavirus [11]. For instance, peptides derived from the VP6 protein have demonstrated efficacy in diagnostic assays. Likewise, peptide-based ELISA assays for Porcine Reproductive and Respiratory Syndrome (PRRS) have exhibited sensitivities ranging from 97.6 to 100% [19].

The insufficiency of diagnostic tools presents a significant obstacle to effectively managing the global spread of ASF. There is an urgent need for a cost-effective and reliable diagnostic solution. This study focuses on the p72 protein of ASFV, a major structural protein that demonstrates high expression levels during infection, constituting approximately one-third of the virion's protein content [20]. Given its conservation and immunogenicity across ASFV genotypes, the p72 protein was utilized as the basis for selecting eight synthetic peptides using immunoinformatic tools. Experimental validation of these peptides' diagnostic potential involved dot-ELISA and indirect-ELISA assays using rabbit hyperimmune serum specific to recombinant p72 protein. A previous work demonstrated the use of peptides in developing an ELISA test for infectious bronchitis virus, yielding a coincidence ratio of 95.4% compared to a whole virus-based ELISA test [21]. Similarly, other studies have

efficiently utilized bioinformatic tools to develop indirect ELISA assays for precise antibody identification against ASFV [22]. While previous studies have focused on various proteins like p54, K205R, and CD2v to identify B cell epitopes in ASFV [23–25], this study uniquely employed the p72 protein of ASFV with bioinformatic tools to identify B cell epitopes. The process involved a two-tier protein scanning mechanism utilizing the Bepipred 2.0 prediction tool in the first step to identify potential antigenic regions within the p72 protein. These regions were then further analyzed using BCEpred and ABCpred in the second step. This two-stage approach integrated analyses from different indices and artificial network-trained algorithms, resulting in the shortlisting of eight potential peptide antigens for ELISA.

Field samples collected during outbreaks of ASFV were categorized as either positive or negative, and their validity was confirmed using a commercially available kit, yielding consistent outcomes. Peptides P1, P3, P4, and P7 were identified through ROC analysis as exhibiting high sensitivity and specificity. The developed ELISAs showed promising results in detecting antibodies against the p72 protein of ASFV. Only one negative sample was found positive result in ELISAs using peptide 1 and peptide 2, while it tested negative in the remaining six peptide ELISAs. The sample was tested negative in the Biostone ELISA kit. The sample was obtained from an unaffected region, which could potentially result in a false positive due to the reduced specificity of the two peptides. Previous studies utilizing proteins such as p17, p30, and p72 have also demonstrated potential in serodiagnostics [26–28], aligning with the peptide-based findings of this study. The p72 peptide-based ELISA exhibited promising outcomes and demonstrated its suitability as a convenient diagnostic tool for detecting ASFV antibodies. The developed assay demonstrates no cross-reactivity with the relevant viral pathogen that presents similar clinical symptoms, such as CSF. The newly developed peptide-based indirect ELISA will suit well for serosurveillance studies and detecting ASFV infection in pig population.

Conclusions

Overall, creating a highly accurate and effective serological diagnostic ELISA is vital for detecting ASFV infection and managing its transmission. Through immunoinformatic tools, we have identified eight distinct immunodominant peptides. Among them, peptides P1, P3, P4, and P7 displayed promising diagnostic capabilities in dot-blot assay and indirect ELISA, with P1 showing the highest immunoreactivity. Comparison with a commercial ELISA kit demonstrated strong correlation. This peptide-based ELISA assay aims at antibody-based ASFV detection, seromonitoring, and serosurveillance. It bridges the gap between precision and feasibility, enhancing disease management in the swine sector. The newly developed p72 peptide-based assay accelerates outbreak identification and improves ASF infection detection in real-world scenarios, offering societal benefits. In summary, utilizing immunoinformatics for selecting immunodominant peptides, presents significant potential to transform ASF infection diagnosis. This research lays the groundwork for enhanced and effective detection of ASFV infection.

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Availability of Data and Material Not Applicable.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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