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Development of Indirect and Sandwich ELISA for Sero-surveillance and Detection of Swinepox Virus

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ABSTRACT

Background: Swinepox is an economically important, classical pox disease of piglets. The present study was undertaken with a view to develop rapid serological tests to diagnose the disease.

Methods: During the study period, 25 suspected swinepox outbreaks in Assam were confirmed by polymerase chain reaction with sequencing and phylogenetic analysis, further the outbreaks were confirmed by transmission electron microscopy (TEM) for identification of swinepox positive samples. The positive samples were used to isolate the virus in PK-15 cell line and develop indirect and sandwich ELISA.

Result: The cell culture-based indirect ELISA was developed that demonstrated an accuracy of 88.8% compare to VNT and 100% sensitivity with 66.67% specificity, could identify 61.71% seroprevalence of swinepox in random pig serum samples. A sandwich ELISA was also developed with polyclonal sera raised in rabbits as coating antibody and swinepox positive pig serum as tracing antibody. The sandwich ELISA detected 77.78% positive cases compared to PCR. Swinepox is an emerging disease in North-eastern region with high sero-positivity observed during random sampling. This is the first report of using immune sorbent assays to detect swinepox.

Key words: Antigenic detection, Development, Indirect, Sandwich ELISA, Sero-surveillance, Swinepox virus.

INTRODUCTION

Pig rearing is a vital occupation of rural society, especially the tribal people of Assam. But certain diseases like swinepox hinder the growth of the pig and affect the economy of the rural farmers.

Swinepox is a worldwide disease, which is host-specific for pigs and occurs in young piglets up to 4 months of age (Afonso *et al*., 2002). The etiological agent is an enveloped DNA virus of the *Poxviridae* family with immunologically distinct characteristics.

Swinepox usually spread by direct contact; however, congenital transmission is also possible (Paton *et al*., 1990). The association of poor sanitation and/or intensive breeding with open-herd management and presence of the hog louse (*Hematopinus suis*), which is the main mechanical vector (House and House, 1994), aggravate the disease. Other vectors of the Swinepox virus (SWPV) are biting flies (*Stomoxys calcitrans*) and black flies (*Simuliidae*) that mechanically transmit the disease.

Recovered animals are immune to re-infection with SWPV (Proietto *et al.,* 2016). No vaccines are available so far against swinepox.

Diagnosis of swinepox is limited to clinical signs and outbreak history, including isolation of the virus and electron microscopy. Sero-diagnosis of the disease is confined to agar gel diffusion precipitation tests and immune-electro-osmophoresis test. Some workers (Riyesh *et al*., 2016) have also used PCR and sequencing for the diagnosis of swinepox.

The present investigation was undertaken with an aim to develop immunosorbent assays to detect swinepox in serum or tissue scabs and also to characterize the circulating SWPV in Assam. To the best of our knowledge, this is the first attempt to identify the swinepox by indigenously developed indirect and sandwich ELISA.

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MATERIALS AND METHODS

Sample collection

Suspected outbreaks of swinepox in Assam were recorded from 2016 to 2017. From the infected animals, (about 1-3 months of age, of either sex) scab and serum samples were collected. Random serum samples of in-contact pigs in the farms or from the vicinity of the suspected outbreak areas,

including serum samples of pigs from Mizoram and Arunachal Pradesh were collected and used.

PCR confirmation of swinepox

Viral DNA was isolated from the specimens using QIAamp DNA mini kit (Hilden, Germany) and quantify.

Polymerase chain reaction (PCR) was performed targeting amplification of *SPV120* gene including a portion of *SPV119* gene of SWPV using specific primers (Table 1). PCR was carried out in 50 µl reaction volume using *Pfu* DNA polymerase (Thermo Scientific). The concentration of the template used for the PCR reaction was 37 ng/µl. The PCR was set with 95°C for 5 minutes as denaturing temperature, 30 cycles of denaturing at 95°C for 1 minute followed by annealed at 52°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 5 minutes. A product of 755 bp was visualized in 2% agarose gel.

Sequencing and Phylogenetic analysis of swinepox based on extracellular enveloped protein gene

Amplified products of PCR were purified and TA-cloning was done using pMD20-T vector (Takara; Cat. #6028) after appending the poly A tail in the PCR product. Further, the vector was sequenced and sequence analysis was done using online and offline software.

Phylogenetic analysis was done by Neighbor-Joining (NJ) method using the molecular evolutionary genetics analysis version nine (MEGA 9) software. Selected identical extracellular envelop gene sequences of other poxviruses were retrieved from GenBank for comparative analysis.

Electron microscopy of swinepox suspected scab

For further confirmation of SWPV infection, four scab samples were sent to the Sophisticated Instrument Facility unit (SAIF), North Eastern Hill University, Shillong, Meghalaya for TEM and electron micrographs were visualized.

Isolation of swinepox virus in laboratory, PCR confirmation and virus quantification

PK-15 cell line was used for virus isolation. For *in-vitro* infection with SWPV, approximately 5×10^5 cells were seeded in 25 cm^2 tissue culture flask to attain 60-70% confluency on the day of virus inoculation. A cell suspension of 10% (W/V) of 300 µl was used for inoculation. The infected flasks were maintained with a 2% maintenance medium.

Virus titer was determined *in vitro* by calculating the Tissue Culture Infectious Dose (TCID) using the procedure of Reed and Muench method (1938); a series of 10-fold dilutions of the original virus sample was made and TCID50 titer was determined as 10^3 $TCID_{50}$/50 µl in 3 days.

Development of cell culture-based indirect ELISA for sero-surveillance study of swinepox

After confirmation of swinepox by PCR and determining the virus titer, the cell culture supernatant (after 5th passage) was freeze-thawed and centrifuged at 5000 rpm 4°C for 10 minutes and then the supernatant was heat-inactivated at 56°C for 30 minutes (Ouchi *et al*., 1988) for its further use as coating antigen of Indirect ELISA with carbonate-bicarbonate buffer. Pre-blocking was done with 3% LAH (50 µl/well). After incubation with primary antibody (Swine infected/ convalescent serum) (50 µl) (1:50) in 3% LAH, anti-pig IgG HRPO-conjugated secondary antibody was added to each well. For detection, freshly prepared chromogen-substrate mixture was added and the reaction was stopped by 50 µl of stop solution (1M H_2SO_4). The plate was read at 490 nm in a plate reader.

Samples, showing OD_{490} value as double or more than double the mean of negative serum control (using bovine serum samples as negative control) were considered as positive for SWPV (Positive serum control range: 0.30-1.20; Negative serum Control range: 0.07-0.20; Conjugate Control and Blank OD below 0.07).

Virus neutralization test (VNT)

Serum samples used for ELISA development were tested for neutralizing antibodies to SWPV (Teppema and De Boer, 1975). Briefly, after inactivation, sera were serially diluted in DMEM (1:2 and 1:4) in 96-well plates in duplicates. A SWPV suspension containing 10^3 $TCID_{50}$/50 µl volume was added to each well and incubated at 37°C in a 5% CO_2 incubator. Subsequently, approximately 3×10^4 PK-15 cells/ wells were added and the plates were incubated until viable CPE were observed in the wells. Results were expressed as positive or negative on the basis of detection/ neutralization of virus-induced CPE.

Development of sandwich ELISA for antigenic detection of swinepox

An in-house Sandwich Enzyme-Linked Immunosorbent Assay (s-ELISA) was developed to measure the SWPV antigen in the scabs. The cell culture supernatant was used (procedure; head 2.6 first paragraph) to raise hyperimmune serum against SWPV in rabbits. (Three months old, New Zealand white, male rabbits) using the protocol mentioned in Table 2. Sufficient titer was obtained as compare to initial days of blood collection received after the 28th day of injection with a dilution of 1:500 of the serum in 3% LAH in coating buffer.

This polyclonal serum diluted 1:500 in 3% LAH in coating buffer was used as the coating antibody in

Table 1: PCR primer designed for identification of Swinepox virus DNA.

Name	Sequence 5'→3'	Total no.	Ref.	Amplicon size
Env-F	CCATATACACTACAATAAAATCAAA	25	NC_003389.1	755 bp
Env-R1	CCTTTAGTACAGGCACTAGG	20		

Table 2: Procedure for raising swinepox virus hyperimmune serum in rabbits.

Day of injection	Quantity and composition of inoculums	Route	Blood collected for detection of antibody
Zero day	0.5 ml SWPV antigen+0.5 ml Complete adjuvant mixed	I/M	0.5 ml
7th day	1 ml plain heat inactivated SWPV antigen	I/M	0.5 ml
14th day	1 ml plain heat inactivated SWPV antigen	I/M	0.5 ml
28th day	1 ml plain heat inactivated SWPV antigen	I/M	0.5 ml

MaxiSorp® plates. Unpurified 10% tissue suspension was used to detect the SWPV antigen. Known positive serum (iELISA positive serum sample and scab from the same animal positive in SWPV PCR) from the natural outbreaks were used as tracing antibody in 1:50 dilutions. 50 µl of anti-pig IgG Horse-Radish Peroxidase (HRPO) conjugate diluted at 1:500 in ELISA blocking buffer was used. For detection, freshly prepared chromogen-substrate mixture was added and the reaction was stopped by stop solution (1M H_2SO_4). Absorbance of individual wells was measured at 490 nm in an ELISA reader. The results were interpreted as corrected OD values obtained by subtracting the OD value of background wells from the OD value of test wells. The difference of OD value ≥0.1 was considered as positive.

Statistical analysis and graphs

Chi-square test was used to determine significant difference among the results in different diagnostic tests, using the software MedCalc v18.5 for Windows (MedCalc Software). Further Receiver-operating characteristic (ROC) curve, the area under the curve (AUC), calculation of positive and negative predictive values (PPV and NPV, respectively) and the agreement test (inter-rater agreement-κ coefficient) were conducted with the help of the same software to determine the sensitivity and specificity of the tests. The κ coefficient was calculated to determine the magnitude of the statistical agreement between cell cultures based on indirect ELISA and VNT.

RESULTS AND DISCUSSION

A total of 25 outbreaks were recorded. Altogether 54 scab samples and the same number of serum samples of the affected pigs were collected. A total of 64 serum samples from recovered animals as well as 30 random serum samples were also collected for sero-surveillance. For random surveillance of swinepox antibody, 62 serum samples of pigs, (37 samples from Mizoram and 25 samples from Arunachal Pradesh) were used.

Out of 54 scab samples collected, 52 (96.30%) were found to be positive for SWPV by PCR with samples showing a product of 755 bp (Fig 1) on 2% agarose gel electro phoresis.

Sequencing of cloned plasmid and Phylogenetic analysis

Out of the PCR positive samples, four samples were purified, cloned, sequenced and sequence analysis was done. The *SPV120* complete nucleotide sequences were submitted to GenBank and accession numbers KY973402, KY973403, MG983833 and MG983834 were assigned. Further, the

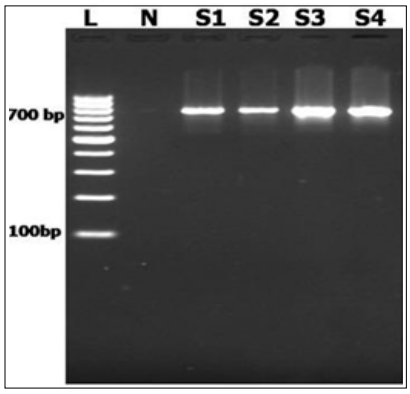


Fig 1: SWPV Swinepox virus confirmation by PCR.
*Note: L-ladder 100 bp, N-mplate Control, S1 to S4- Sample.

sequences were aligned with the *SPV120* gene sequence of SWPV (ALF07850.1) present in the NCBI database using ClustalW. The result showed that there was nucleotide substitution at various positions in the sequences and in the position 402-404, there was insertion of CAA trinucleotide. Further, the gene sequence was converted to the predicted amino acid sequence and aligned with ALF07850.1 amino acid sequence to observe its alteration within the sequences using ClustalW 2.1. The results showed that there was N-Asparagine amino acid (codon CAA) insertion in position 138. The samples of SWPV were sequenced and subjected to similarity analysis. The results showed that the SWPV *SPV120* gene sequences shared a very close relationship with other SWPV strains from India (KR028365.1) and USA (AF410153.1) with nucleotide sequence identities from 99~100%. The percentage of identity shown by the sequences with selected identical extracellular envelop gene sequences of other poxviruses retrieved from GenBank was 12~63%.

Phylogenetic analysis was done using MEGA9 software by the Neighbor-Joining (NJ) approach (Fig 2). Selected identical extracellular envelop gene sequences of other poxviruses were retrieved from GenBank for a comparative analysis (Table 3). Phylogenetic analysis showed that the present SWPV isolates from Assam are similar to the previously reported Indian isolates and placed within the same cluster.

Transmission electron microscopy (TEM) for identification of swinepox positive samples

The TEM result showed that the virions measure 100-450× 110- 260 nm with a core surrounded by an outer coat. In the cytoplasm of the affected cells, mature and immature virus

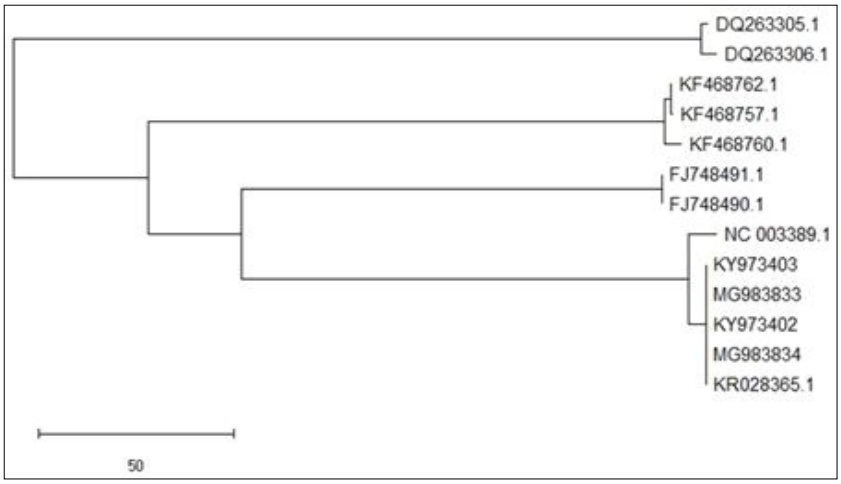


Fig 2: Phylogenetic analysis of different poxviruses based on the nucleotide sequences of envelop protein gene.
*Note: Bootstrap test (500 replicates) are shown next to the branches.

Table 3: Nucleotide sequences of envelop protein gene of other pox viruses obtained from NCBI database used for phylogenetic analysis.

Virus	Gene	Country/isolate	NCBI accession numbers
Swinepox virus	SPV120	VTCC isolate/India	KR028365.1
Swinepox virus	SPV120	USA	NC_003389.1
Goat Pox	P32	Maha/goat/19/India	KF468762.1
Sheep poxvirus	P32	Maha/goat/22/India	KF468760.1
Goat poxvirus	P32	JK/goat/27/India	KF468757.1
Buffalo pox virus	D8L	India	FJ748491.1
Buffalo pox virus	D8L	India	FJ748490.1
Orfvirus	B2L	67/04/India	DQ263305.1
Orfvirus	B2L	79/04//India	DQ263306.1

particles including lamellar bodies were observed (Fig 3). Inside the virions, the characteristic lateral body was seen. Some cells contained multiple layers of membranes particles. This indicated the presence of SWPV in the infected tissue.

Isolation of SWPV in PK-15 cell line and quantification

In this study, the PK-15 cell line was used. From the second blind passage onward, the cellular changes were noted (Fig 4) and these changes were clearer from the third passage onward. The cytopathic effect included rounding of the infected cells, the cell membranes of nearby cells were fused and they coalesced to form unique structures (Syncitia). The large cells were visible after 48 hours of virus inoculation. After 72 hours of infection, the cells were more rounded and the normal architecture started disappearing and about 96 hours after infection, the cells were completely detached from the surface of the culture flask.

After the third passage of virus inoculation, the flasks showing CPE were used for viral DNA extraction and PCR confirmation of Swinepox virus. The amplified product was observed on 2% agarose gel, results showed (Fig 5).

Development of cell culture-based Indirect ELISA for identification swinepox antibody in serum

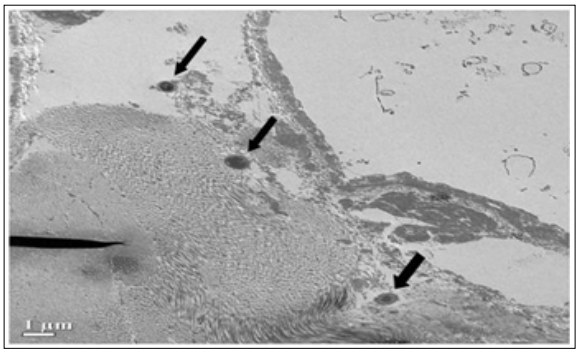
In the study, the sero-prevalence of SWPV was found to be 65.24% (137/210) (Table 4). Out of 54 outbreak serum samples, 51 (94.44%) were found positive for SWPV. A total of 64 suspected swinepox recovered serum samples were collected during the study period, of which 39 (60.94%) were found positive for SWPV antibody. Interestingly, out of 92 random samples, 47 (51.09%) were positive for SWPV by indirect ELISA.

sELISA for detection of SWPV antigen from the skin scabs

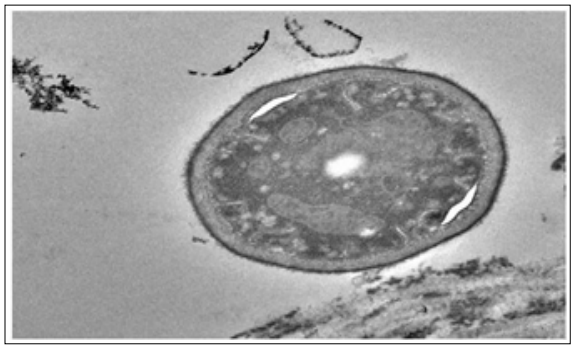
In the present experiment, tissue suspension of 54 suspected swinepox scabs was used for detection of SWPV antigen. Swinepox positive serum samples that were previously confirmed by iELISA and scab samples positive by PCR were used as tracing antibody. As a negative antigen control, 10% chicken tissue suspension was used. Out of 54 scab samples tested, 42 (77.78%) were found positive for s-ELISA.

VNT

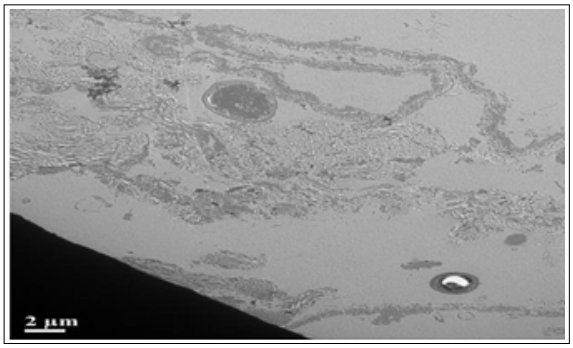
In the present study, 210 pig serum samples were evaluated by VNT, of which 125 (59.53%) were found positive (absence of CPE) and all were considered to be truly positive in the indirect ELISA. Among the 85 VNT negative (CPE observed in the dilution 1:2) serum samples, 12 were considered to be false negative since the ELISA readings showed values above the cut-off.



a: Virus particles within the cytoplasm of the cells.



b: Lateral body with lamellar structure of the swinepox virion.

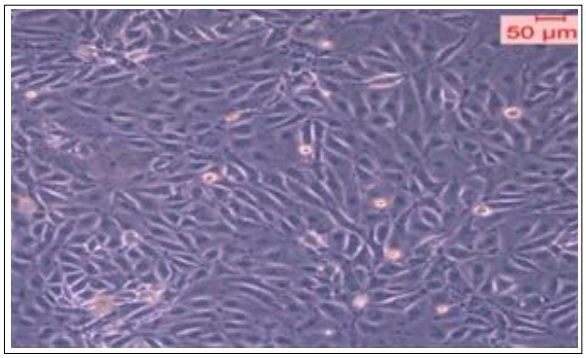


c: Devoid of cellular structure vicinity of the virus particles

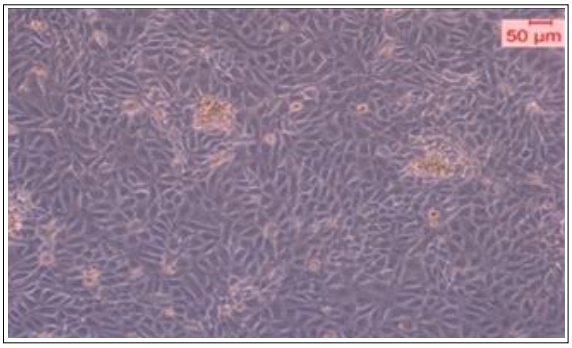
Fig 3: Electron micrograph showing the swinepox viral particle structures at different magnification.

Table 4: Results of SWPV indirect ELISA.

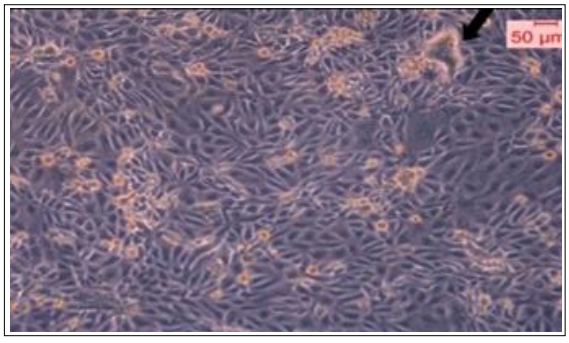
Place		Sample used for ELISA		
		Outbreak serum	Recovered animal serum	Random animal serum
ASSAM	Kamrup (R)	15	13	9
	Kamrup (M)	27	42	20
	Lakhimpur	9	9	1
	Karbi Anglong	3	-	-
Mizoram		-	-	37
Arunachal Pradesh		-	-	25
Total (No.s)		54	64	92
			156	
Overall		210		



a: Healthy PK-15 monolayer.



b: PK-15 monolayer after 24 hrs of SWPV infection.



c: PK-15 monolayer after 48 hrs of SWPV infection.

Fig 4:PK -15 cell line healthy vs infected with SWPV.

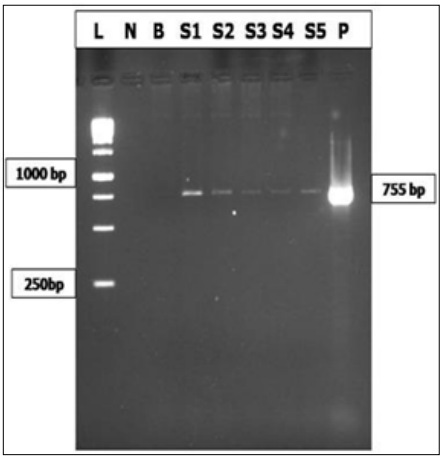


Fig 5: SWPV PCR positive cell culture isolates in 2% agarose gel. *Note Here: L- ladder 1 kb, N-No Template Control, B-Blank, S1-S5- Cell culture isolate, P- Positive control.

Table 5: Statistical analysis of results by chi-square test.

Tests	Positive	Negative	Row totals
SWPV PCR	52 (48.00) [0.33]	2 (6.00) [2.67]	54
iELISA	51 (48.00) [0.19]	3 (6.00) [1.50]	54
sELISA	42 (48.00) [0.75]	12 (6.00) [6.00]	54
VNT	47 (48.00) [0.02]	7 (6.00) [0.17]	54
Column totals	192	24	216 (Grand total)

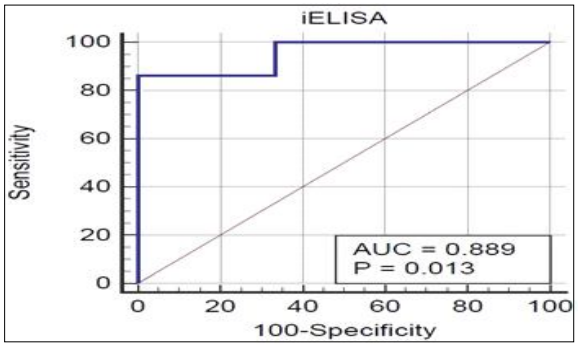


Fig 6: Receiver-operating characteristics (ROC) curves based on test results obtained for the iELISA (*n* = 210).

Statistical analysis

The Chi-square value was found to be 11.625 (*p*-value < .008785), indicating a significant difference among the tests (Table 5).

Compared to the results of the VNT, the AUC indicated that the developed iELISA was 88.90% accurate (Fig 6). The 95% confidence intervals (CI) of the AUC for the iELISA ranged from 41.3 to 99.9% and the significance level (Area = 0.5) was P<0.013. The sensitivity of the test is 100% and specificity 66.67%.

Positive predicted values (PPV) and negative predictive values (NPV) were 91.24% (95% CI 86.01-94.64) and 85.88% (95% CI 77.87-91.32), respectively, in a population with a disease prevalence of approximately 61.71%. The κ coefficient between VNT and indirect ELISA was found to be 0.614 (95% CI 0.409-0.819).

Swine pox is a prevailing pig disease in the Assam/ North Eastern Region of India. Based on sequencing and phylogenetic analysis, it was established that the circulating SWPV of Assam is similar to other reported Indian isolates.

This seems to be the first report of using a cell culture-based Indirect ELISA for sero-surveillance of SWPV infection. The indigenously developed ELISA could identify the prevalence of swinepox approximately as 61.71%.

The Sandwich ELISA developed during the present study could be used successfully to detect SWPV infection using scab samples and it could be an alternate way to identify SWPV in such cases, where collection of serum is not possible.

Lack of proper diagnostic tools to identify the etiology of the disease increases the case fatality rate. In this aspect, the developed iELISA or sELISA may be useful for detection of swinepox which may help to control and prevent the disease.

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Conflict of interest: None.

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