

## Development and Evaluation of an Antigen Capture Enzyme-Linked Immunosorbent Assay (AC-ELISA) for the Diagnosis of African Swine fever

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Received date: May 25, 2015; Accepted date: Aug 06, 2015; Published date: Aug 10, 2015

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### Abstract

African swine fever (ASF) is a viral haemorrhagic fever of pigs with devastating impact on pig production and household income in Africa. Lack of a vaccine and treatment for ASF has increased the dependence on accurate diagnosis as basis for control and possible eradication of the disease. The aim of this study therefore, was to develop and evaluate an in-house sandwich enzyme linked immune sorbent assay (ELISA) using antibodies raised against African swine fever virus (ASFv) isolates from Uganda for diagnosis of ASF. The ASFv was grown in pig alveolar macrophages, the infected cells harvested, lysed and the virus precipitated using polyethylene glycol 6000. The virus was purified on Sepadex G-200 column equilibrated with 50mM Tris-HCl PH 7.2 containing 0.15M NaCl and viral proteins separated by SDS-PAGE. The target protein (vp73), was quantified and used to immunize rabbits to produce polyclonal antibodies against it. The purified immunoglobulin IgG (rabbit anti ASF-vp73) was used for antigen capture in sandwich ELISA. Eighty eight (88) known positive and 176 known negative pig serum samples were used to evaluate assay performance. The diagnostic sensitivity of the ELISA was 82.95% (95% CI, 78-100%), diagnostic specificity was 96.59% (95% CI, 90-100%). Positive and negative predictive values were 92.4% and 91%, respectively. The inter samples coefficient of variation of raw optical density values for known positive samples in different runs was <10% (range 1.1-7.8), while intra sample coefficient of variation ranged from 0.6-5.5% between runs. The developed antigen capture ELISA has a high diagnostic sensitivity and specificity and is therefore good for detection of active ASF infection. However, the developed assay should be further validated using larger sample size under different laboratory conditions and referenced serum samples from different ASF endemic countries.

**Keywords:** African swine fever; Polymerase chain reaction; Antigen capture; Enzyme linked immunosorbent assay; Diagnostic sensitivity; diagnostic specificity

### Introduction

African swine fever (ASF) is a highly infectious viral haemorrhagic disease of pigs that poses a high potential devastating risk to pig production in the world and Africa in particular [1]. In Uganda ASF is endemic and sporadic outbreaks of the disease have been reported in all regions of the country [2,3]. Several diagnostic tests have been developed to detect ASF in infected pigs, and these include: polymerase chain reaction [4], indirect ELISA [5], immunoblot, immunofluorescence [6], immunohistochemistry and *in situ* hybridization [7] and haemadsorption test [8]. Each of the diagnostic technique has its strength and weakness.

The indirect Enzyme linked immune sorbent assay (ELISA) is one of the most sensitive and reliable antibody detection diagnostic assays widely used in the diagnosis of [9,10]. However, it tends to give a high number of false positive results with field sera and it requires regular standardization of the procedure in different laboratories. To address these concerns, Hutchings et al. [11] recommended that samples to be

used in ELISA should be diluted at least double the initial volume to minimize false positives. Furthermore, ELISA positive serum samples should be retested using other serological tests such as immunoblot, immuno-fluorescent antibody test so as to confirm the results [12]. Repeated freeze thawing of samples has been shown to reduce nonspecific reactions in antigen capture ELISA and this was also reported to improve the specificity of the indirect ELISA as the numbers of false positives were reduced [11]. Polyclonal rabbit anti-ASFV antibodies used as antigen capture immunoglobulins and guinea pig anti-ASF vp73, as ASF antigen detector were effectively used to diagnose ASF [11]. Nevertheless, the limitation of antigen ELISAs is the high detection limit, hence samples with low antigen concentration (in-apparent or chronic ASF) may show false negative [11].

Identification of highly antigenic ASF viral protein in infected cells is important in the development of diagnostic immune-assays [12]. Over 100 polypeptides are known to be induced by ASFV in infected mononuclear phagocytic cells and about 50 of these proteins are antigenic [10]. Forty of the polypeptides are incorporated into viral particles [13]. Among the ASF viral capsid proteins, vp73 is highly immunogenic and has been used in several studies to detect antibodies against ASF virus using indirect immune-assays. Other immunogenic ASF capsid proteins that have been used in antigen capture ELISAs

include: vp54, vp30, and vp17 [14]. Previous studies and observations suggested that antibodies induced against vp54 recognize linear epitopes of the target antigens [12]. Further investigation by Gallardo and others [14] suggested that sero-detection of vp54 and vp30 in poorly preserved samples was not effective. This could be associated with loss of antigenicity or very low levels of these proteins in poorly preserved samples. Furthermore, ELISA recognizes conformational epitopes in fresh and well preserved samples; however, conformational epitopes structure in poorly preserved samples is lost, hence reducing sensitivity of ELISA to detect antigens in the later type of samples [15].

Recombinant viral protein vp54 was shown to be a reliable candidate for use in sero-diagnosis of ASF in West Africa [12]. However, when recombinant vp54 based assay was compared with the OIE approved ELISA to diagnose ASF, the former had lower sensitivity in detecting infection with the East African ASF isolates [16]. Furthermore, Malin [17], while investigating an outbreak of ASF in Mityana District of Uganda, reported that out of the 18 pigs that were found positive for ASF viral genome using RT-PCR, only two were sero-positive when a commercial indirect ELISA was used.

Vidal et al. [18] developed an improved solid phase ELISA using monoclonal antibodies raised against ASF viral protein VP73 and reported that the assay was sensitive and specific. The developed sandwich ELISA was able to detect lower antigen levels at a concentration of 0.05 µg/ml of the VP73. This was lower than the detection limits of 0.6 µg/ml obtained when polyclonal antibodies were used to develop similar ELISA. The team was able to detect the whole ASF viral particles using monoclonal antibody based assay to a limit of  $2.3 \times 10^2$  PFU/ml. They then concluded that indirect ELISA was a vital assay in epidemiological survey of ASF, while the direct ELISA that utilizes monoclonal antibodies against specific viral proteins is effective in the detection of ASF viral antigens.

Immunological antibody detection techniques are routinely used and easy to perform. However, these assays are unable to detect early stages of viral infection in acutely infected animals in which antibody response has not been elicited [19]. This fact was demonstrated by Gallardo et al., [20]. The team tested porcine serum samples from central Uganda, where they noted that antibody detection performed using the OIE prescribed ELISA and immunoblotting assays where all negative for antibodies against ASF viral antigen. However, PCR was able to detect ASF virus in the same blood and tissue samples that were negative for ASF when OIE indirect ELISA and immunoblot assay were used. ASF virus was isolated from at least four serum samples which were non-reactive for antibodies against ASF virus. This finding reconfirms the previous studies that described a low detection rate of ASF virus infection in pigs from East African countries using both recombinant antigens and OIE prescribed ASF serological methods [21].

Nucleic acid or ASF viral antigen detection are a suitable option for diagnosis of ASF since viremia occurs early in ASFv infection and persists for a long time, contrasting the indirect immunoassays where antibody formation and detection require at least a week post-infection [22,23]. Moreover, sometimes pigs die before antibodies are formed, especially in hyper-acute ASF infection. Although PCR is highly sensitive and specific, it is expensive and only limited to research settings in many developing countries including Uganda [20,24]. Therefore, there was a justifiable need to develop and introduce a cheaper, sensitive and specific antigen detecting technique using viral antigen derived from Ugandan isolates of ASF virus to complement

antibody assays in the country. Early detection of ASF would enable institution of timely and effective control measures against the disease.

The aim of this study therefore, was to develop and evaluate an in-house ASF viral antigen capture ELISA using antibodies raised against purified viral proteins vp73 derived from a local (Ugandan) isolate to detect ASFv antigens in the country. The sensitivity, specificity, positive and negative predictive values including the repeatability of the assay was evaluated.

## Materials and methods

### Culture and Purification of ASFv

African swine fever virus isolated from an infected pig in Uganda was cultivated in porcine alveolar macrophages using established protocol [25]. A poly ethylene glycol-600 (PEG-600) virus purification protocol modified after Abcam Viral Purification kit, Olaho-Mukani, personal communication [25] was used for first stage antigen purification. The technique involved lysing ASF virus infected alveolar macrophages that showed maximum cytopathic effect by repeated freezing and thawing followed by centrifugation of the lysate at 3200 ×g at 4°C for 15 minutes to remove the cell debris and nuclei. The supernatant was collected and onto every 20 ml of the viral suspension was added 5 ml of 25% PEG-6000 solution and incubated at 4°C overnight. The mixture was centrifuged at 3200 ×g for 30 minutes at 4°C, the supernatant carefully aspirated and discarded, while the white viral pellet was re-suspended in 2 ml of 0.1M PBSpH 7.2 (virus re-suspension solution). Trace amounts of PEG were removed by the addition of one volume of solution containing 4 M KCl and 50 mM tris-HCl, pH 7.2 to three volumes of the concentrated virus suspension and left on ice for 30 minutes. The suspension was centrifuged at 12,000 ×g at 4°C for 10 minutes to precipitate PEG and salts. The virus-rich supernatant was collected and aliquots stored at -80°C for subsequent procedures.

### Further Purification of ASF Virus on Sephadex G-200 Column

Just before ASF virus antigens were separated by SDS-PAGE, the virus suspension was further purified on a gel chromatography column filled with sephadex G-200. In brief, the virus suspension was dialysed overnight against one liter of 50 mM Tris-HCl pH 7.2 containing 0.15M NaCl. Sephadex G 200 was also equilibrated in the same buffer. A 2.5 cm diameter by 120 cm long chromatography column was packed with the sephadex G-200 and washed four times by passing through virus elution buffer (0.15M NaCl+50 mM Tris-HCl pH 7.2). This was followed by addition of 5mls of virus suspension into the column and eluted with the same buffer. Fractions of 10 ml of the eluent were collected and presence of viral protein in the fractions detected using Bradford assay and the optical density (OD) of the solution measured at 595 nm [26]. The ASF protein-rich fractions were pooled, concentrated by osmosis using PEG-6000 pellets and the concentrated virus suspension stored at -80°C for subsequent procedures.

### Separation and Quantification of ASFV Proteins

Purified ASFv suspension was sonicated and solubilized in buffer containing 20 mM Tris-HCl, 5 mM EDTA-Na, and 1x protease inhibitor, pH 7.4 at 4°C for 30 minutes, and the proteins separated by SDS-PAGE [27]. The viral proteins were separated using 12%

separating gel at constant voltage of 200 V for 70 hours. The bands were stained with Coomassie brilliant blue (R 250) over night. The gel was then de-stained in two changes of de-staining solution one composed of 40% methanol and 10% acetic acid in distilled water. Further de-staining was done in second solution composed of 5% methanol and 7% acetic acid in distilled water. The concentration of the separated viral envelop protein, Vp73 was estimated using densitometer. In brief the samples containing the target viral proteins were electrophoresed in 12% 0.5 mm polyacrylamide gel and the gel stained with Coomassie brilliant blue (Coomassie brilliant blue G- 250, Applichem GmbH, Germany, Lot No 2L002872) and gently agitated for two hours to enable the dye penetrate into the gel. The gel was then de-stained in four changes of a solution composed of 40% methanol, 10% glacial acetic acid in distilled water for 48 hours. The intensity of the dark blue dye corresponding to particular protein band was measured by quantitative ultraviolet scanning densitometry technique [28] using densitometer version GS 800.

### Confirmation of the Presence of the Target Viral Proteins vp73 by Western Blot Technique

The viral proteins of interest in ASF virus suspension were demonstrated by western blot technique based on the protocol described in OIE manual for terrestrial animals [10]. The procedure involved transfer of the SDS-PAGE separated proteins on to nitrocellulose membrane (Immune-Blot™ PVDF membrane Bio-Rad Labs Hercules CA). The transferred protein bands on the membrane were stained with 0.1% Ponceous stain in acetic acid to visualize and evaluate the effectiveness of protein transfer on to the membrane. The membrane was labeled with numbers corresponding to the loaded wells and cut into strips each strip containing distinct target protein bands. The membrane strips were then de-stained with distilled water through several washes until the protein bands disappeared and the membrane strips cleared of the stain. The unbound sites on the membrane strips were blocked by incubating them in skimmed milk overnight. The excess protein and skimmed milk were washed off in three changes of 0.01M PBS pH 7.2 containing 0.05% Tween twenty (T-20). Primary antibody (anti vp73) was added and incubated at room temperature while shaking for one hour. The unbound antibody was washed off followed by addition of goat ant rabbit IgG peroxidase conjugates (diluted 1:5000 in 0.01M PBS pH 7.2) and incubated at room temperature for an hour. The membranes were washed thrice in PBS, T-20 and substrate chromogen (DAB) was added and left at room temperature on shaker for 5 minutes. The membrane was washed in tape water, dried and the result read. Distinct brown bands indicated the presence of the target protein were identified against the set markers.

### Production of Rabbit anti- ASF vp73 Polyclonal Antibodies

Polyclonal antibodies against ASF vp73 were raised by intramuscular inoculation of the together with Freund's adjuvant (FCA&FIA, Sigma Aldrich Co. USA) into 2 rabbits and boosted three times to achieve optimal antibody titer. In the first immunization, each rabbit was administered intramuscularly 300 µg (1.2 ml) of the prepared ASF viral antigen in Freund's complete adjuvant at six sites (0.2 ml per site) on the back region of the animal. The small quantity of the antigen administered per site was to minimize irritation and chances of necrosis occurring at the site of deposition of the antigens. Booster doses (0.2 ml per site) of 300 µg ASFV antigen in Freund's incomplete adjuvant was administered intramuscularly at six sites after

14 days post immunization and repeated at intervals of two weeks. The rabbits were bled every 10 days post inoculation (pi), serum obtained and presence of antibodies in the serum monitored using western blot. Serum that contain antibodies against ASF antigen was diluted serially and each dilution was titrated against the selected ASF viral protein (Vp73) to get the end point dilution that was used to estimate the concentration of antibodies in the serum of each immunized rabbit. At optimal antibody titer, final bleeding was done and antibodies isolated and purified.

### Isolation, purification and quantification of polyclonal antibodies

Isolation of nonspecific immunoglobulin from serum was carried out by sequential protein fraction steps as previously described [29]. The process involved precipitation of Gamma globulins from the serum of the immunized rabbits in 45% ammonium sulphate, followed by gel filtration using Diethylaminoethyl cellulose (DEAE-C Sigma Aldrich Co. UK) in column chromatography packed and equilibrated with 0.005M PB pH 7.8 and immunoglobulin fractions eluted with the same buffer at room temperature. Fractions of 10 ml of the eluent were collected and presence of proteins (immunoglobulins) in the fractions tested and the protein-rich fractions that had antibodies were pooled and concentrated by osmosis using PEG-6000. The amount of immunoglobulins in the purified and concentrated solution estimated using established procedure [26,30].

### Conjugation of rabbit ant ASFV protein (vp73) to horse radish peroxidase

Several methods for conjugation of enzymes to antibodies have been described and the efficiency of each procedure differ [31,32]. Most of the methods documented couple enzymes to amino groups of immunoglobulins [32]. In this study rabbit anti-ASF-vp73 conjugation was achieved by application of a modified protocol of Wilson and Nakane [33,34]. The procedure involved addition of 5 mg of horse radish peroxidase (HRP, Type VI-S, Sigma Aldrich Co. USA) to 0.9 ml of double distilled water, followed by addition of 1ml of 0.1M sodium-iodoperoxidase (NaIO<sub>4</sub>) at concentration of 32 mg/ml. The mixture was stirred at room temperature for 30 minutes followed by dialysis against 1mM acetate buffer, pH 4.4 at room temperature with two changes of the dialysate each for two hours. Further dialysis was done at 4°C over night with three changes of the dialysate. 1 ml of rabbit ant ASFV p73 polyclonal antibody (4 mg/ml) was mixed with 1 ml of carbonate- bicarbonate buffer, pH 9.6, followed by addition of the periodate-oxidised antibody, the mixture adjusted to pH 9.0 and stirred gently for 2 hours at room temperature. The pH was adjusted to 7.6 and the solution left at 4°C over night without stirring. Two milligrams of glycine was then added to the solution and stirred gently at room temperature for 2 hours. The antibody conjugate was dialysed against 0.01M PBS, pH7.2 at 4°C over night, followed by centrifugation at 2000 xg at 4°C to remove debris.

The supernatant was collected and to it was added equal volume of glycerol and 1.0 ml aliquots of the conjugate stored at -20°C till needed for use in the next procedures.

### ASF antigen capture (sandwich) ELISA

Sandwich ELISA was carried out to detect ASF viral antigens using a protocol described by Huchings et al. [11]. Optimal titers of polyclonal capture antibody; rabbit anti-vp73 IgG conjugate and serum dilutions

were determined by checkerboard titration. Briefly, polystyrene coated high binding micro well plates (Nunc MaxiSorp<sup>®</sup>, Bioscience) were used for developing ELISA. Nunc MaxiSorp ELISA plates were coated with prepared capture antibody (anti vp73) at optimal dilution of 1/3200 in sodium carbonate and bicarbonate buffer solution at optimal PH 9.6 and temperature 37°C. The plates were incubated at 37°C for 3hrs, followed by incubation at 4°C overnight. The unoccupied hydrophobic sites on the plates were blocked using 1% gelatin in PBS. The unbound proteins were washed off using 0.01 M PBS pH 7.4 containing 0.05% Tween 20, followed by the addition of pig serum that contain the target antigen at optimal dilution(1/40), the plates were incubated at 37°C for an hour and the solution discarded and wells washed thrice with PBS. Then 100 µl per well of rabbit anti ASF vp73 IgG peroxidase conjugate diluted optimally at 1/1600, was added and plates incubated at 37°C for an hour. The plates were then washed thrice with PBS containing Tween 20 as previously described. Substrate chromogen, TMB (Nacalai Tesque Inc., Nakagyo-Ku, Kyoto 604-0855 Japan) was added and plates incubated at room temperature for 5 minutes, the reaction was stopped by addition 100 µl per well of 1.0M orthophosphoric acid and OD values of the solution in wells of the plates read at 450 nm in a micro spectrophotometer [11].

### Data quality control

As quality control and to improve the reliability of the results, three positive, negative and blank control samples were included in each ELISA plate in every run of the assay. Every sample was diluted two folds to reduce background effect as reported in previous studies [11]. Nonspecific reaction was reduced by repeated freeze thawing of samples as this practice is known to have had no effect on the OD values of ELISA [11].

### Assay performance evaluation

The performance of the developed ELISA was evaluated based on established procedures [35,36]. True positive (TP), True negatives (TN), False positive (FP) and false negative (FN) test outcomes including assay sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were used in assessing the assay performance. In evaluating ELISA results, optical density (OD) value >0.1 above background was considered positive reaction for ASF antigen and that ≤ 0.1 was considered negative.

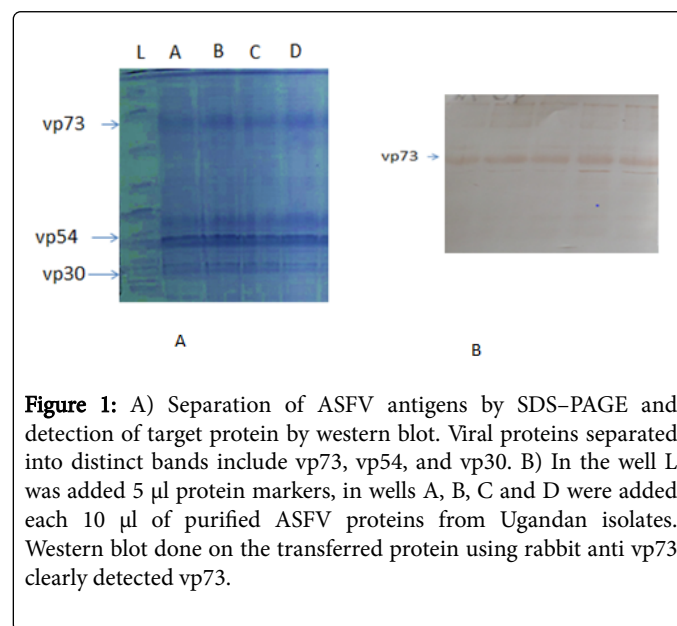
### Ethics statement

Full ethical clearance was obtained from the Uganda National Council for Science and Technology (UNCST) and from the College of Veterinary Medicine, Animal Resources and Bio-security, of Makerere University under reference number VAB/REC/11/110. Animal welfare and care was ensured in accordance with the international Guideline on Animal Welfare and Euthanasia. Any experimental animal in pain or moribund was immediately euthanized to relieve it from further suffering. Clean water and commercial feed were provided ad libitum to all pigs during study period.

## Results

### Separation of ASF viral proteins and confirmation of vp73 by western blot

African swine fever viral proteins were separated by SDS-PAGE using 12% separating gel. Several proteins of different molecular weights were separated among which three proteins were prominent i.e. vp73, vp54, vp30 as shown in Figure 1a. The highly immunogenic viral protein vp73 was demonstrated by western blot technique (Figure 1b).

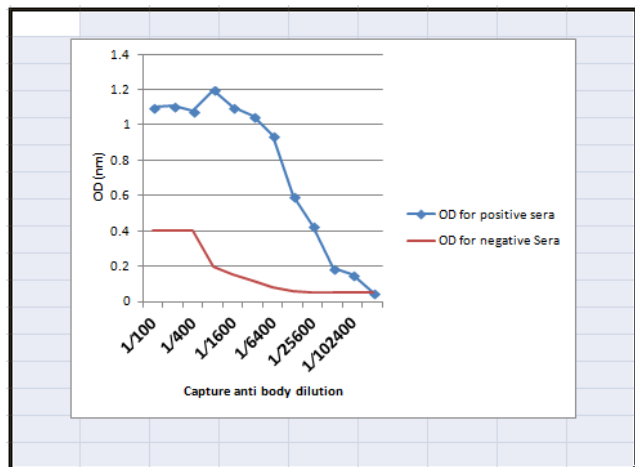


**Figure 1:** A) Separation of ASFV antigens by SDS-PAGE and detection of target protein by western blot. Viral proteins separated into distinct bands include vp73, vp54, and vp30. B) In the well L was added 5 µl protein markers, in wells A, B, C and D were added each 10 µl of purified ASFV proteins from Ugandan isolates. Western blot done on the transferred protein using rabbit anti vp73 clearly detected vp73.

### Optimization of the reagents for antigen capture ELISA

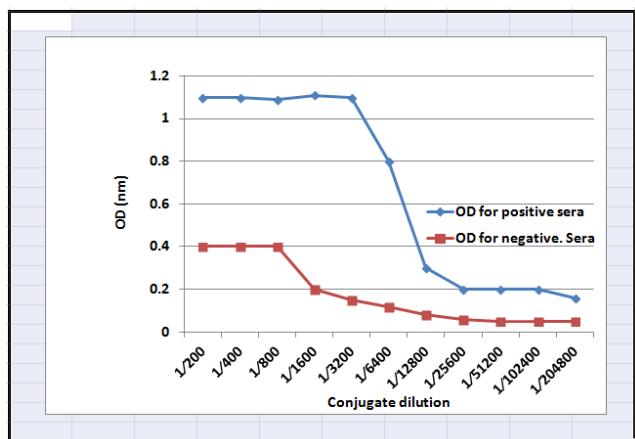
The capture antibody (rabbit anti-ASFvp73) was titrated concurrently with the conjugate while the dilutions of the reference positive and negative sera were kept constant at 1/40. Optimal capture antibody dilution was 1/3200 (Figure 2).



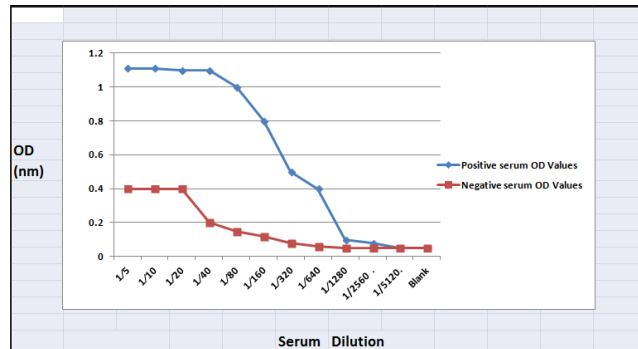


**Figure 2:** Determination of the optimal capture antibody dilution: 1/3200 was the optimal antibody dilution.

And that for conjugate was 1/1600 (Figure 3). The known positive and negative sera were titrated while the concentrations of capture antibody and the conjugates were kept constant at 1/3200 and 1/1600, respectively. The finding showed that the optimal dilution for the known positive and negative sera that had minimum background effect was 1/40 (Figure 4).



**Figure 3:** Determination of optimal conjugate dilution: 1/1600 was the optimal conjugate dilution.



**Figure 4:** Determination of optimal Serum Dilution: 1/40 dilution was the optimal dilution

### Evaluation of the performance of the developed ELISA

A total of 246 pig serum samples were used to evaluate the performance of the antigen capture ELISA developed. All samples were tested for ASF virus DNA by means of conventional diagnostic PCR using OIE prescribed primers. Diagnostic PCR revealed that, 88 samples had ASF viral DNA, hence confirmed positive samples and 176 samples were known negative samples. Of the 88 known positive samples, the developed assay tested 73 as true positives and 15 PCR positive samples tested negative (false negatives). Out of 176 known ASF virus negative samples, 170 tested negative (true negative) while six tested positive (false positive).

Chi square statistical analysis showed that the diagnostic sensitivity (Dse) was 82.95% (at 95% CI, 78–100%) and diagnostic specificity (Dsp.) was 96.59% (at 95% CI, 90–100%). The positive predictive value (PPV) and negative predictive value (NPV) were 92.4% and 91.9%, respectively. The findings were as summarized in Table 1.

### Evaluation of the repeatability of the developed ELISA

To evaluate repeatability of the assay, each sample was run as replicates in each test and between runs. Raw absorbance values were used as indicators of the test analyte for the developed ELISA.

		Reference samples of known infection status			
		Infected Animals		Un infected Animals	
Test Results	Positive (+)	73	TP	FP	6
	Negative (-)	15	FN	TN	170
		Dse=73/73+15= 82.95%		Dsp=170/170 +6=96.59%	

**Table 1:** Two by two (2 × 2) tables for determination of association between infection status and test results.

Four runs of each sample in duplicates were done in four separate tests and coefficients of variation (CV) for raw absorbance values were calculated to evaluate repeatability. The coefficient of variation (CV) of raw optical density (OD) value within a sample run repeatedly four times was less than 10% and the range of intra sample variation was

1.1–7.8% for known positive samples. Inter sample variation between the four runs ranged from 0.6% to 5.5%. On the other hand for known negative samples, the coefficient of variation within samples was 2.7–12.3% and inter negative sample CV ranged from 3.3% to 10.6% (Table 2).

Number of Runs	Sample Status							
	OD values for Known positive sample			CV (%)	OD values for Known negative samples			CV (%)
	1	2	3		1	2	3	
1	1.084	1.134	1.086	2.1	0.030	0.037	0.035	10.6
2	1.102	0.998	1.100	4.6	0.031	0.030	0.033	4.9
3	1.110	0.980	1.094	5.5	0.029	0.031	0.030	3.3
4	1.100	1.089	1.104	0.6	0.030	0.028	0.032	6.7
CV (%)	1.1	6.1	7.8		2.7	12.3	6.4	

**Table 2:** Raw optical densities and coefficient of variation within and between samples in repeated runs.

## Discussion and conclusion

Several diagnostic assays are available for ASF and a number of them are being used worldwide. These include immunofluorescence [6], polymerase chain reaction [4], immunohistochemistry and *in situ* hybridization [7], haemadsorption test [8], loop-mediated isothermal amplification, LAMP [37]. Despite the existence of these assays, diagnosis of ASF in many regions of Africa where the disease is endemic is often based on clinical-pathologic techniques which are tentative diagnostic methods. This is probably because most of the available diagnostic techniques are expensive and cumbersome. More so confirmatory diagnostic techniques for ASF are designed for use in reference, regional or national laboratories [22]. Though rapid antibody diagnostic assays have been developed and validated for onsite detection of ASF, they are not routinely used as diagnostic tool in many African countries including Uganda where the disease is endemic. This could be due to the fact that some of the immune assays such as the available OIE recommended commercial antibody ELISA has been reported not to detect some of East African isolates of ASFV [23]. Worse still, antibody assays are effective only in sub-acute, chronic or subclinical ASF where the course of the disease is long to allow for antibody production.

In this study we developed antigen detecting in-house ELISA for diagnosis of ASF. Contrary to the late antibody response to ASF virus infection in pigs, viremia often occurs early in ASF and persists over a long period of time. This makes nucleic acid or antigen detection techniques a suitable option for ASF diagnosis, unlike indirect immunoassays where antibody formation and detection require at least a week post infection [21,22]. Although PCR is highly sensitive and specific, it is expensive hence limited to research facilities in many developing countries including Uganda [19,23]. There was therefore a justifiable need to develop and introduce cheaper pen side antigen detecting technique to complement antibody assay. Early detection of ASF would permit institution of timely and effective control measures for such devastating diseases. Antigen capture ELISA is known to be

effective diagnostic tool for ASF and the result were even better at the peak of clinical disease [11].

Furthermore previous studies demonstrated that, the performance of diagnostic ELISA that use polyclonal antibody as antigen capture immunoglobulin was high [11]. Hutches et al. [11] then suggested that ELISA that uses rabbit anti-ASF virus antisera as capture antibody with a detector could be used as confirmatory diagnostic assay for ASF, based on its high specificity and sensitivity. In the current study, the sensitivity and specificity of the antigen capture ELISA were 82.95% and 96.59% respectively. These values are high and concur with earlier reports [11]. Though the suggestion by Hutches et al. [11] to use ELISA as confirmatory diagnostic assay for ASF was based on results of a study from ASF virus infected cell culture where the viral load is often higher than in natural infection, our finding concurred with the previous studies because of the high sensitivity and specificity of the developed assay in this study. The findings of this study together with previous ones emphasize the importance of sand which ELISA in detection of ASF viral antigen.

In the current study the in-house antigen capture ELISA based on ant-vp73, had high diagnostic sensitivity and specificity (82.95% and 96.59% at 95%. The positive predictive value (PPV) and negative predictive value (NPV) were 92.4% and 91.9% respectively. Contrary to the high performance of antigen ELISA on Ugandan pig serum samples, Gallardo et al. [38] reported that recombinant p54 based ELISA had low sensitivity on pig sera from East Africa as opposed to sera from West Africa and Europe. Gallardo et al. [19] further reported a low sensitivity of the OIE prescribed ELISA and immunoblot assays in diagnosis of ASF in Uganda. Though the performance of the in-house ELISA in this study was high, the assay failed to detect 17.05% of the known positive samples as positive and 3.41% known negative samples as negative. This could be due to the acknowledged limitation of antigen ELISA which is the high detection limit [11]; hence samples with low antigen concentration (in apparent or chronic ASF) could have contributed to the 15 false negative results. The 5.01% false positive test results could also be associated with nonspecific reactions.

Positive and negative predictive values of any diagnostic assay are important in evaluating the feasibility of screening programs and in assay evaluation [39]. The assay developed in this study was able to detect 92.4% of the known positive samples as positive and 91.9% known ASF virus negative samples as negative. These values of positive and negative predictions of the assay test out comes were higher than in the earlier reports [38].

To evaluate repeatability of the antigen capture ELISA, we ran each sample as replicates in each test and between runs. The coefficient of variation (CV) of raw optical density (OD) value within known positive sample run repeatedly four times was lower than that of the known negative samples 1.1–7.8% and 2.7–12.3% (acceptable CV value <20% [40,41], respectively. Inter sample variation between the positive samples in the four runs ranged from 0.6% to 5.5% while that of the known negative samples ranged from 3.3% to 10.6%. Jacobson [40] showed that three to four replicates of each sample used in assay validation in at least five plates on separate runs sufficiently provided preliminary estimates of repeatability of the assay. For assay to be considered to have adequate repeatability “the coefficient of variation should generally be less than 20%”, if the coefficient of variation is more than 30% in majority of the samples used for assay validation and or between runs of the assay, preliminary studies should be repeated [40]. In this study the intra samples and inter known positive samples coefficient of variation of raw optical density was less than

10%. The CV within known negative samples was slightly higher than that of positive sample and the peak CV was 12.3%. However, these values of CV of raw OD of the samples used to evaluate the assay in the current study were less than the 20% limit recommended to consider diagnostic assay to have adequate repeatability [40]. Diamandis and Christopoulos [42] stressed the importance of coefficient of variation in evaluating precision of immunoassays and further stated that coefficient of variation is applicable only when standard deviation of the figures is proportional to the mean. Furthermore, Rodbard [43] recommended that intra and inter assay coefficient of variations should be calculated for appropriate assay evaluation and the values are often used as quality controls in qualitative laboratory assays.

We concluded therefore, that the developed antigen capture ELISA has high diagnostic sensitivity and specificity hence could be used to augment PCR as a reliable diagnostic assay for ASF in Uganda. However, the developed assay will be further validated using a larger sample size from other geographical areas.

## Acknowledgement

This study was funded by the Millennium Science Initiative, under the Uganda National Council of Science and Technology through a grant to Prof. Lonzy Ojok, Dr. William Olaho-Mukani and Dr. Julius Boniface Okuni of the Appropriate Animal Diagnostic Technologies project. Supplementary funding for completion of the research work and publication of the output was obtained from school of research and Graduate studies, Makerere University, Kampala. The authors acknowledge the technical guidance of Dr. Yoshikazu Iritani, a Japanese virologist and technical expert to Uganda on JICA project for improvement of diagnostic services in the country who gave technical guidance during ASF virus isolation and pathogenicity study. Special regard to the management and staff members of Central diagnostic laboratory, College of Veterinary Medicine animal Resources and Biosecurity, Makerere University, Uganda, for providing conducive environment to accomplish this research. We are grateful to Mr. Kelvin Muwonge and Mr. Phillip Kimuda of the Molecular Biology Laboratory, COVAB, Makerere University who gave technical back stopping during protein separation and confirmation.

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