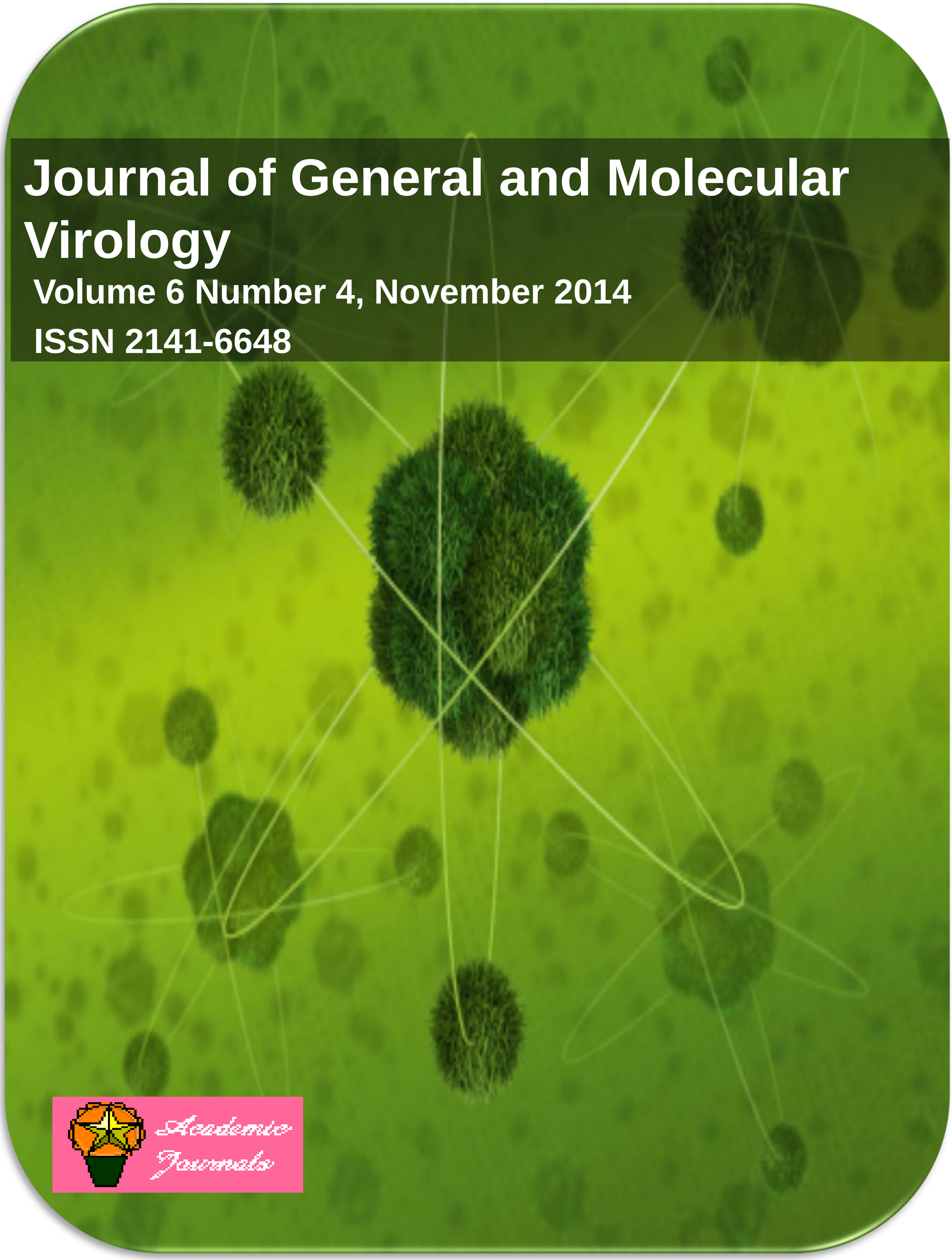

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Journal of General and Molecular Virology

*Full Length Research Paper*

# Comparison of immunoperoxidase monolayer assay, polymerase chain reaction and haemadsorption tests in the detection of African swine fever virus in cell cultures using Ugandan isolates

**Mathias Afayoa[1], David Kalenzi Atuhaire[1,2], Sylvester Ochwo[1], Julius Boniface Okuni[1], Kisekka Majid,[1] Frank Norbert Mwiine[1], William Olaho-Mukani[3] and Lonzy Ojok[1]***

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**African swine fever (ASF) is a devastating viral disease of pigs and is among the major hindrances to pig industry in sub-Saharan Africa including Uganda. The aim of this study was to compare immunoperoxidase monolayer assay (IPMA) to PCR in detection of ASF virus in infected macrophage cultures and to categorize ASF viral isolates in Uganda by haemadsorption assay. Field strains of ASF virus were isolated from infected pigs into swine alveolar macrophages culture. The effect of the inocula on the cell culture was monitored daily and the presences of ASF virus in the inoculated macrophages were detected using PCR and IPMA. The isolates were then categorized by haemadsorption assay. 58.8% of the samples had ASF virus DNA and ASF virus was isolated from 27% of the samples. IPMA detected ASF viral antigens in 80% of the inoculated macrophages culture 48 hours post infection compared to the 100% by PCR. 95% of the virulent ASF viral isolates from Uganda were haemadsorbing. This study makes the first attempt to use IPMA and haemadsorption assay for the detection of ASF virus and categorization of the African swine fever virus (ASFv) field isolates into haemadsorbing and non-heamadsorbing in Uganda, respectively. The study demonstrates that IPMA is an appropriate option to PCR and could be used to detect ASF virus in cell cultures. It is recommended that the genome of the non-haemadsorbing ASF viral isolates could be sequenced and compared with that of haemadsorption (HAD) isolates to identify molecular peculiarities and markers of these two categories of ASFv.**

**Key words:** African swine fever (ASF), African swine fever virus (ASFv), Immunoperoxidase Monolayer Assay (IPMA), swine alveolar macrophages, haemadsorption (HAD) Polymerase Chain Reaction (PCR).

## INTRODUCTION

African swine fever (ASF) is a highly contagious haemorrhagic disease of domestic pigs caused by a large icosahedral DNA virus that belongs to genus *Asfivirus* and family *Asfarviridae* (Dixon et al., 2005). The disease has devastating effect on pig industry in Africa and is the major setback to pig production particularly in Uganda.

The disease has been reported annually in different regions of Uganda during the last ten years (Rutebarika and Nantima, 2002; Atuhaire et al., 2013).

Currently, there is neither vaccine nor treatment for ASF; the only control strategy for the disease is early detection of the disease followed by instituting strict disease control measures such as quarantine measures and movement control (Solenne et al., 2009). Laboratory diagnostic methods for ASF include viral isolation, detection of ASF viral genomic DNA, detection of viral antigens in porcine tissues and detection of antibodies against ASF virus antigens in serum (Wilkinson, 2000).

In endemic areas, serologic diagnosis is frequently used while in regions where the disease is newly introduced, it is preferable to detect the virus DNA or antigens. Methods which have been used for ASF virus detection include the long-established haemadsorption test (Malmquist and Hay, 1960), immunofluorescence (Colgrove et al., 1969), polymerase chain reaction (PCR) (King et al., 2003; Aguero et al., 2003, 2004), and recently LAMP (James et al., 2010). In Uganda, however, standard confirmatory diagnostic techniques are not in place in many laboratories hence, suspected ASF cases are diagnosed principally on the basis of clinical signs and postmortem lesions. Molecular diagnostic techniques of ASF in many countries have been limited to research only (Gallardo et al., 2011; Tejlar, 2012; Atuhaire et al., 2013). This is due to lack of appropriate diagnostic facilities and reagents in these countries (Oura et al., 2012). Lack of readily available and reliable diagnostic tests in many developing countries often delays the institution of effective ASF control measures; hence farmers often incur enormous losses through pig mortalities and loss of market for pigs and pig products.

Immuno-assays are often used in diagnosis of ASF, however, the OIE recommended ELISA has been reported not to detect some of the East African strains of ASF virus (Gallardo et al., 2011), hence the need for a more sensitive and readily available techniques for the confirmation of ASF in this region. Both *in situ* hybridization and immunocytochemistry have been compared and evaluated for localization of ASF virus in infected cells as a prerequisite for their use in the diagnosis and studies of the pathogenesis of ASF in domestic pigs, warthogs and bush pigs (Oura et al., 1998). Recent immunohistochemical studies carried out in Uganda to determine the prevalence of African swine fever viral antigens in slaughter pigs at Wambizi abattoir, Kampala revealed that of the slaughtered pigs with lesions suggestive of ASF, 0.1% had ASF viral antigens in their tissues (Ssajjakambwe et al., 2011).

Viral isolation is one of the sensitive diagnostic methods; however it requires special bio-containment facilities and a source of swine monocytes and macrophages (OIE Manuel for Terrestrial animals, 2012). The isolated ASF viruses can be categorized by HAD and characterized by molecular techniques such as PCR.

Pig macrophages and monocytes *in vitro* are cells suitable for cultivation of ASF virus as wild type ASFV isolates do not replicate in conventional cell cultures. This is because ASF virus naturally infects and replicate in mononuclear phagocytic cells (Malmquist and Hay, 1960; Sanchez–Torres et al., 2003). In mononuclear phagocytic cells *in vitro*, ASF virus mimic natural infection and most strains of the virus grow readily in monocytes and macrophages culture (Carolina et al., 2010). These cells are frequently used in ASF viral isolation and haemadsorption diagnostic tests. Use of other cell lines for ASF virus cultivation and plaque formation assays require cell culture adapted virus strains (Carolina et al., 2010). Pulmonary lavage can produce sufficient yields of alveolar macrophages that can be used for ASF viral culture and titration (Bustos et al., 2002). The culture medium should be supplemented with serum of a pig from which the alveolar macrophages were obtained (Bustos et al., 2002). In many laboratories, bovine fetal serum is added to cell culture medium to supplement macrophages, however, Bastos et al. (2002) reported that addition of bovine fetal serum reduces infective viral particles in macrophages to 10 - 20% when the cell culture is infected with several ASF virus isolates. The team further suggested that, the ability of each particular batch of pig serum to support the production of infective virus should be tested. This is because the viral yield could differ between the alveolar cell stocks.

Polymerase chain reaction (PCR) is a specific diagnostic test that detects genomic DNA in body fluids, tissue samples and can be used even when the samples are unsuitable for virus isolation and antigen detection (Aguero et al., 2003). Although PCR is a reliable diagnostic assay, it requires specialist equipments and the risk of cross contamination is high (Oura et al., 2012). There is therefore a need to evaluate other cheap, reliable, affordable and readily available diagnostic assays for detection of ASF.

The aim of this study was to compare IPMA to conventional PCR in detection of ASF virus in infected cell cultures and to categorise ASF viral isolates in Uganda based on their ability to cause haemadsorption to infected macrophages. This was because IPMA and

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PCR can detect both haemadsorbing and non haemadsorbing isolates as opposed to HAD test that only detect pathogenic haemadsorbing ASFv. IPMA is an immunelogical test that is based on the principle of the specific binding of antibodies to antigen. It detects ASF viral protein in fixed cells, tissues and is less expensive as it does not require specialist equipments unlike PCR.

## MATERIALS AND METHODS

### Study design

This was a prospective comparative study in which IPMA was compared against conventional PCR, (used as a reference test in this study) in detection of ASF virus in cell culture. ASF virus was isolated from field and experimental samples. Pig tissue samples were collected for viral isolation. Field ASF viral isolates were obtained from all the four major regions of the Uganda, namely, Northern, Eastern, Southern and Western regions. From each region at least 20 sets of tissue samples from dead or clinically sick pigs were collected depending on the frequency of outbreaks of ASF in each region. Stored samples from experimentally infected pigs in previous studies were also included in this study. The sample were then screen for ASF virus using conventional PCR, samples that had ASF virus were processed and filtrate used to inoculate swine macrophages culture. The cytopathic effects of the inoculums on the macrophages were monitored daily and presence of ASF virus in the culture was comparatively confirmed using PCR, IPMA and HAD assay. The details are as described under the subsequent subheadings.

### Sample collection, preparation and ASF virus isolation in pig macrophages culture

Tissue samples for viral isolation were collected from 148 carcasses of pig suspected to have died of ASF or clinical cases. Of these, 136 samples were from the field cases and 12 were preserved samples from experimentally infected pigs. Samples collected for viral isolation included spleen, haemorrhagic lymph nodes, pharyngeal tonsils and kidneys. The samples were screened for ASF viral DNA using PCR and tissues that had ASF viral DNA were macerated, PBS then added. The suspension was sheaved through sterile gauze and filtered through 0.45 µ Whatman filter. Viral isolation was done in pig alveolar macrophages which were harvested from 4-5 months old healthy pigs based on procedure described elsewhere (Carrascossa et al., 1982). The viability of the harvested macrophages was evaluated using trypan blue dye exclusion technique (Strober, 2001) and cell viability of above 90% was used for viral isolation. The viable macrophage suspension was then transferred into six-well culture plates and incubated at 37°C in 5% $CO_2$ for 24 h. To the macrophage cultures, 100 µl of the sample filtrate (viral suspension) was added per well in six well plates. The inoculated plates were incubated at 37°C, 5% $CO_2$ for one hour to enable viral adsorption to take place. This was followed by addition of growth medium (EMEM) containing 5% pig serum to the inoculated macrophages and incubated under the same conditions. The effect of the inoculums on the cell culture was monitored daily for cytopathic effects (CPE). The success of ASF virus isolation was confirmed by PCR and IPMA. ASF virus isolates were characterised based on the ability of the virus to induce haemadsorption on the infected macrophages.

### Immuno-peroxidase monolayer assay (IPMA) for detection of ASF virus

Immuno-peroxidase monolayer assay for detection of the ASF viral proteins in the infected macrophages was done using a modified protocol described by Direksin et al. (2001) and Liang et al. (2013). In brief, the culture medium in the wells discarded and 1 ml of 10% buffered formalin containing 1% Nonidet P40 (NP40) was added and the plates were incubated at room temperature for 30 min to allow thorough cell fixation. The fixed cells were then washed three times using 0.5% Tween 80 in PBS to remove excess fixative. The primary antibody used was lyophilised hyperimune pig serum raised against ASF viral antigens in experimental pigs (Kindly given by Dr. Gallardo from Spain). It was diluted 1:400 in PBS (PH 7.2) containing 2.5% pig serum and 600 µl of the diluted antibody was added to each well and incubated for 30 min at 37°C. The wells were then washed thrice followed by addition of 600 µl of anti pig IgG peroxidase conjugate diluted 1:600 in 0.5% Tween 80 in PBS and incubated at 37°C for 30 min. The wells were then washed with PBS (pH 7.2). Finally, 600 µl of substrate, 3-amino-9-ethyl carbazole (AEC) solution was added to each well and incubated at room temperature for 30 min. The cells were then examined under inverted microscope to evaluate the IPMA results. Red intracytoplasmic staining of the macrophages was considered positive for ASF virus antigens. Non specific staining was considered doubtful and repeated, while unstained cells were considered negative for IPMA, hence not infected by ASF virus. PCR was also used to confirm ASF viral isolation alongside IPMA.

### Haemadsorption (HAD) test

Haemadsorption test was done based on modification of the established protocol (Malmquist and Hay, 1960). In brief, the pig alveolar macrophages culture was prepared, seeded in six well plates and inoculated with ASF virus isolates as earlier described. To prevent non specific haemadsorption; the serum and the alveolar macrophages used were from the same pig. The inoculated plates were incubated at 37°C in 5% $CO_2$ atmosphere. 600 µl of 0.5% freshly prepared pig erythrocytes in buffered saline was added onto the inoculated cells per well and cultures were then examined for haemadsorption daily for 5 days. The result was considered positive when pig erythrocytes adhere to the surface of the infected macrophages forming a ring or clusters. It was declared negative when neither haemadsorption nor CPE occurred in inoculated cell cultured. Infection by non haemadsorbing ASF virus was suspected when CPC was observed but no haemadsorption occurred and this was confirmed by PCR and IPMA.

### ASF viral DNA extraction and PCR

ASFv DNA was extracted from pig tissue samples and inoculated cell culture using QIAamp DNA mini kit for blood and tissues (www.qiagen.com/products/dna/qiaamp-dna-mini kit, QIAiamp® DNA and Blood mini handbook 2012). The DNA was eluded in 10 mM tris hydrochloride (PH 7.8, eluent volume of 200µl) and stored at -20°C until testing. PCR was performed using Go Tag® green Master Mix PCR kit (Promega Corporation USA2012). The primers designed against vp 72 region of the ASF virus genome and recommended for detection of ASF DNA by OIE manual for diagnostic tests and vaccines for terrestrial animals (2009) was used, thus (F 5' – ATG GAT ACC GAG GGA ATA GC – 3', R 5' CTT ACC GAT GAA AAT GAT AC – 3'), (Wilkinson 2000). The reaction mixture consisted of 12.5 µl 1x GoTaq®green Master mix,

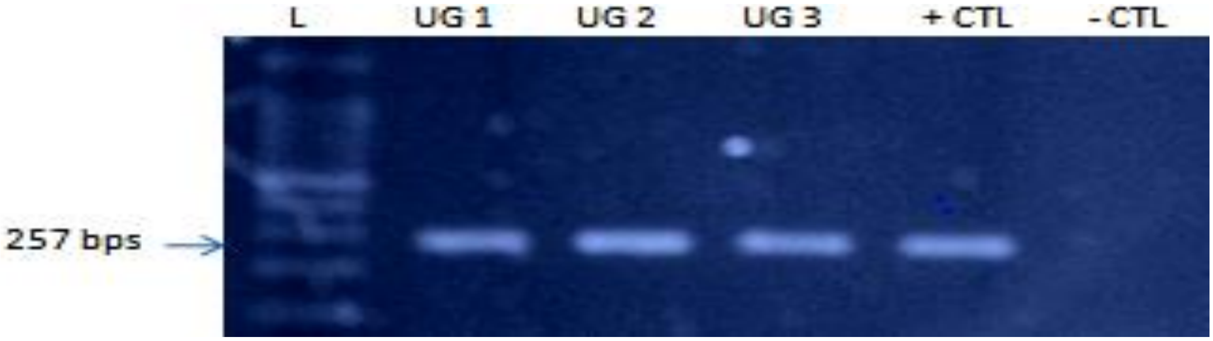

**Figure 1.** Detection of ASF viral DNA from cell culture by PCR. Culture 1 to 3 (Ug1, UG 2 and Ug 3) had ASF viral DNA, hence were all positive, + CTL is positive control culture, – CTL is negative control and L is the DNA ladder.

1 µl of 0.8 µM forward and reverse primers each, 2 µl of DNA template and 8.5 µl of double distilled water in a total reaction volume of 25 µl. The mixture was loaded into and run using MultigeneTM Optimax labnet thermal cycler (Multigene Labnet 99.9°C and temperature accuracy of ±0.5/±0.5. Thermal cycling started at 94°C denaturation 35 cycles each for 30 s, followed by annealing at 50°C and extension at 72°C using reaction volume of 25 µl. PCR products obtained were electrophoresed in 1.2% agarose gel (Nacalai Tesque, inc Kyoto, Japan lot № M2K1602), illuminated by UV system and images were photographed.

### Ethics statement

Full ethical clearance was obtained from the Uganda National Council for Science and Technology (UNCST) and 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

### Sample collection, screening and ASF virus isolation

Out of the 148 pigs autopsied, 12 carcasses were from experimentally infected pigs and 136 were field cases. 69.6% (n =103) of the carcasses had lesions suggestive of ASF and diagnostic PCR done on all the tissue samples revealed that 58.8% (n= 87) of the samples had ASF viral DNA (Figure 1). Of the 87 samples with ASF viral DNA, 82 samples were from pig carcasses that had lesions suggestive of ASF while 5 PCR positive samples were from pigs without lesions suggestive of the disease. More still of the 75 pig tissue samples obtained from the field that had ASF viral DNA, ASF virus was successfully isolated from only 28 samples. On the other hand, ASF virus was isolated from tissues of all the 12 (100%) experimentally infected pigs, making total isolates to be 40 (Table 1).

International inc.), with programmable temperature range of 4 to

### Comparative detection of ASF virus in cell culture by PCR, IPMA and HAD test

To detect ASF virus in the infected cell culture with time, we used conventional PCR and IPMA, comparatively. Viral isolates were then categorized based on their ability to cause haemadsorption in infected macrophages. PCR detected all the 40 (100%) isolates while IPMA detected 32 (80%) of the isolates in macrophages culture within 24 h post infection. However, HAD-test did not show distinct result in the first day post infection in the infected cells. In the second to the fifth day post infection PCR and IPMA detected all ASF virus isolates 100% (n = 40) while HAD detected 95% (n = 38) of the isolates (Tables 2 and 3, and Figure 2).

### Categorization of Ugandan ASF virus isolates using haemadsorption (HAD) test

Of the 40 isolates confirmed by IPMA and PCR, two isolates (5%) were non-haemadsorbing, although they caused clinical disease in experimental pigs and CPE in macrophages cell cultures similar to the rest of the isolates (Figure 3E). Erythrocytes distinctly adhered to the surfaces of the infected macrophages by 48 h post-infection (PI) and by 72 h PI erythrocytes clumped on the remaining intact macrophages (Figure 3F).

### Morphological changes in infected macrophages post inoculation

In the first two days post infection (48 h PI), there were no observable changes in the infected macrophages. However, at 56 h post inoculation (slide C of Figure 4),

**Table 1.** Total number of pigs autopsied and the different tests results.

| Sources of samples | Carcasses autopsied and sampled | Carcasses with ASF-like lesions | PCR positive cases | | Viral isolation |
|---|---|---|---|---|---|
| | | | Carcasses with ASF-like lesions | Carcasses without ASF-like lesions | |
| Field pigs samples | 136 | 91 | 70 | 5 | 28 |
| Experimental pigs samples | 12 | 12 | 12 | 0 | 12 |
| Total | 148 | 103 (69.6%) | 82 (55.4%) | 5 (3.4%) | 40 (27%) |

**Table 2.** Detection of ASF virus in cell culture by PCR, immunoperoxidase monolayer assay (IPMA) and haemadsorption test (HAD) 73 h post inoculation (PI).

| Diagnostic test | No. of isolates | No. positive | Percentage |
|---|---|---|---|
| PCR | 40 | 40 | 100 |
| IPMS | 40 | 40 | 100 |
| HAD | 40 | 38 | 95 |

**Table 3.** Progressive detection of ASF virus in cell culture by PCR, IPMA and HAD

| Diagnostic Assay | Days post inoculation | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| PCR | ++++ | ++++ | ++++ | ++++ | ++++ |
| IPMA | ++ | +++ | ++++ | ++++ | ++++ |
| HAD | -/+ | ++ | ++ | +++ | +++ |

PCR = Polymerase chain reaction, IPMA = immunoperoxidase monolayer assay, HAD = haemadsoption test. + (plus) = Positive test result indicating week detectable ASF virus infection in the cell culture and ++++ strongest positive test result. - (minus) = negative test result, viral DNA, and proteins not detected by a given test at that period

ASF virus infected macrophages were enlarged and more rounded (balloon degeneration). By 72 h post-inoculation (slide D Figure 4) about 60% of the infected cells detached from the surface of the culture plates and were lysed and the intact macrophages were rounded and swollen. The changes are as shown in Figure 4.

## DISCUSSION

In the present study, 69.5% of the sampled pigs had lesions suggestive of ASF; 58.9%, of the samples had ASF DNA. However, lesions due to ASF are not pathognomonic and are often confused with lesions due to other haemorrhagic swine diseases such as classical swine fever, septicaemic salmonellosis, acute trypanosomiasis due to *Trypanosoma Simiae* infection and thrombocytopaenic parpura (Kleiboecker, 2002). To differentiate these haemorrhagic diseases, the use of laboratory test is a prerequisite (Radostits et al., 1995; Aguero et al., 2003). Therefore, pigs that had lesions similar to those of ASF and were diagnosed negative for the disease which could have died of other swine haemorrhagic diseases. This finding therefore emphasises the limitation of relying on clinical and pathologic diagnosis of ASF which happen to be the common practice in developing countries where laboratory diag-nostic services are not readily available. It therefore calls for confirmatory diagnostic capacity to be established to address such suspected cases.

Isolation of ASF virus in this study was done from both field and experimental pig samples. Viral isolation and detection is one of the key diagnostic tests for ASF and in this study, ASF virus was successfully isolated from only 27% (n = 40) of the collected samples, though 58.9% of the total samples collected had ASF viral DNA. This could be attributed to the state at which samples were obtained from the field. Some tissue samples from field were obtained from pigs that had died in the previous day and hence the tissues were partly autolysed. This limited the possibility of isolating ASF virus from such samples. However, PCR was able to detect ASFv DNA in some of the autolysed samples from which viral isolation was not successful. This is contrary to the notion that ASFv is resistant to a number of physical conditions and as it is known to persist in putrefying tissues for several days. On the other hand ASFv were isolated successfully from all samples obtained from carcasses of experimental pigs (n =12) and this was likely due to the controlled environmental conditions in which the experiment was done and samples were collected immediately after death and stored at -20°C awaiting the process of viral isolation. This minimised the chances of viral inactivation, hence good result of viral isolation obtained.

In addition to viral isolation and detection, several techniques have been used to diagnose ASF; these include pathologic diagnosis, immunoassays, PCR and haemadsorption test (HAD). The commercially available diagnostic technologies have varying sensitivities and specificities. In this study, we compared conventional PCR and IPMA which are among sensitive, rapid and

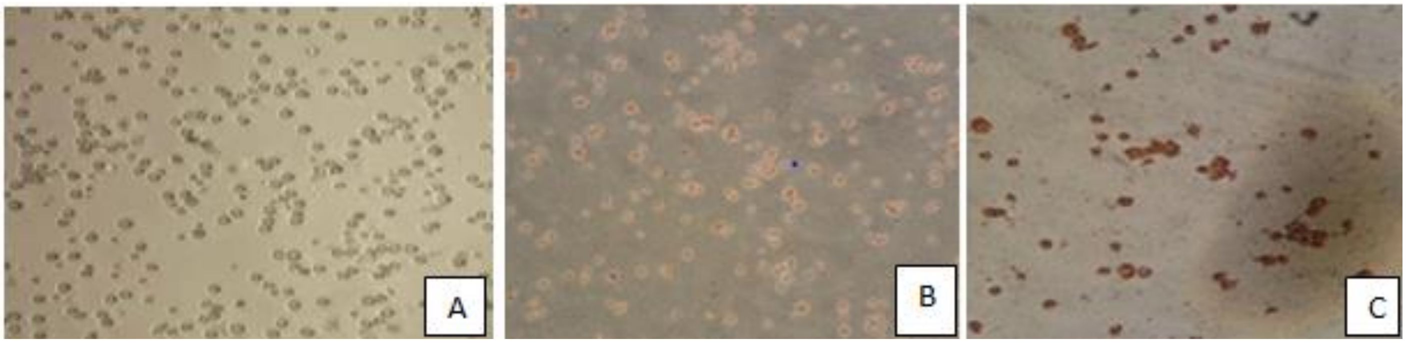

**Figure 2.** Detection of ASF viral antigen from cell culture by IPMA at different time intervals. Culture A is non infected macrophages 72 h PI (negative control), B is ASF virus infected macrophages 48 h PI and C is ASF virus infected macrophages 72.

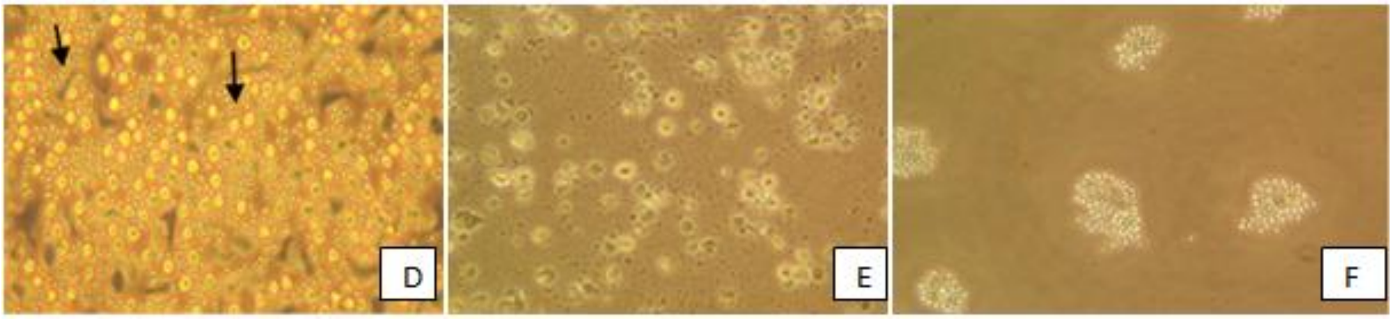

**Figure 3.** Haemadsorption of pig erythrocytes to ASF virus infected macrophages. Slides D and F indicate positive HAD test result 48 and 72 h post–infection, respectively. Slide E, shows negative HAD test of macrophages infected with non-HAD ASF.

specific diagnostic tests (Oura et al., 2002) to detect ASF viral DNA and antigen in ASF virus infected macrophages at different durations post-infection. Previous studies showed that PCR was able to detect all known ASF virus genotypes including that of non-haemadsorbing and less pathogenic isolates. Furthermore it detected ASF genome in degraded or inactivated samples (Oura et al., 2002). Despite the enormous advantages of using PCR as diagnostic test for ASF, it is prone to cross contamination; hence false positive results may occur (Oura et al., 2002). Oura et al. (1998) detected ASF DNA in ASFv infected IBRS2 cells 12 h post infection using in situ hybridisation employing an35S labelled DNA probe. In this study, we found that ASF viral DNA and proteins (antigen) in infected macrophages were at detectable levels within one day post-infection by conventional PCR and IPMA, respectively. PCR detected all the 40 (100%) isolates while IPMA detected 32 (80%) isolates in macrophages culture within 24 h post infection. However, HAD assay did not show distinct result in the first day post infection in some of the infected cells. From the second to the fifth day post infection all the three assays used (PCR, IPMA and HAD) clearly detected ASF viral DNA, antigen and surface adhesive molecules on the infected macrophages, respectively. By the 48 h post infection, PCR and IPMA detected all ASF virus isolates 100% (n = 40) while HAD detected 95% (n = 38) of the isolates. This shows that IPMA can be highly specific in detection of ASF virus in cell cultures. However, PCR was in this case more sensitive than IPMA as the latter detected ASF virus in all the 40 isolates in macrophages culture within 24 h post infection unlike IPMA that detected 80% of the isolates in the same period of culture. Oura et al. (1998) reported that detection of ASFviral protein vp73 using immunocytochemistry was as sensitive as the use of DNA hybridisation and the team noted that, immunocytochemistry assay is a quick, safe and easy diagnostic technique that allows morphological detection of the antigen. Oura et al. (1998) further reported that, attenuated ASF virus isolate infected a high percentage of endothelial cells, alveoli and bone marrow derived macrophages. In this study, we demonstrated that within 24 h post-infection, macrophages infected with Ugandan isolates of ASF virus had detectable amounts of ASF virus proteins and the viral protein concentration increased with time post inoculation. The detection of ASF antigen in infected cells within24 h on this study is in agreement with what was reported by Gallardo et al. (2012), though the later first detected ASF virus 48 h PI in infected COS – 1 cells. The difference in the period of first detection of ASF viral antigen in the infected cells could be due to the different cell types used in the two studies, more so macrophages being the natural target cells for ASF virus unlike COS – 1 cells.

Although earlier studies reported that the OIE recommended ELISA could not detect some of East African strains of ASF virus (Gallardo et al., 2011), the hyper immune serum raised against European (Spain) isolate of ASF virus was able to detect all Ugandan isolates of the virus using IPMA. This was probably because direct IPMA was used to detect ASF viral proteins in cell culture in this study. More so, the viral antigen concentration in infected macrophages culture was probably higher as compared to that in naturally infected pigs.

Majority (95%, n = 38) of ASF virus isolates from Uganda during this study caused adsorption of erythrocytes to infected cells, however 5% of the isolates were non haemadsorbing (non-HAD). Haemadsorption test (HAD) is one of the oldest diagnostic tests for ASF (Malmquist and Hay, 1960) and is based on the fact that porcine erythrocytes adhere to the surface of the infected swine monocytes and macrophages. Most of virulent ASF virus isolates are known to be haemadsorbing and a very small percentage of the isolates are non haemadsorbing (Boinas et al., 2004). Previous studies showed that most of non haemadsorbing ASF strains were a virulent. How-ever, it is well known that a small proportion of non-haemadsorbing ASFv do cause acute disease (Gonzague et al., 2001). This property of haemadsorption could be used as diagnostic assay for haemadsorbing strains of ASF virus in cell culture (Boinas et al., 2004). Specific protein CD2v is responsible for haemadsorbing infected leukocytes (Kay-jackson et al., 2004). CD2v is encoded by a gene EP402R and this protein is similar to adhesion receptor or CD2 on T – lymphocytes (Rodriquez et al., 1993). EP402R gene is reported to be responsible for the adhesion of swine erythrocytes to the infected leukocytes, while EP153R encodes for protein that stabilizes the adhesion molecules (SOP/CISA/ASF/VI/I/2008). In addition to causing HAD, CD2v is also associated with impairment of lymphocytes replication in response to mitogens (Barca et al., 1998). Contrary to what we found in this study, Kay-Jackson et al. (2004) reported that non HAD ASF virus did not cause disease in experimental pigs but induced antibody production that persisted throughout their study period (49 days post infection). The pigs infected with non pathogenic, non-HAD ASF virus isolates were protected against pathogenic HAD virus isolated from the same farm. However, in this study we found that the non-HAD ASF virus isolates were virulent and caused cytopathic effect (CPE) in infected cells by 56 - 72 h post inoculation and they caused clinical disease in infected pigs similar to that due to HAD ASF virus. Our findings were in conformity with what was reported by Gonzague et al. (2001) that showed that some of the non-HAD ASF viral isolates caused high mortalities of 80-90% in domestic pigs in Southern Africa and Madagascar. This shows that phenotypic characteristic of haemad-sorption or non-haemadsorption of ASF virus is not exclusively the determinant of the pathogenicity of ASF isolates. The finding of Gonzague et al. (2001) was similar to what we reported in this study where the non HAD ASF virus isolates were virulent in domestic pigs and caused clinical disease in experimentally infected pigs. This limits the sensitivity of HAD assay in detection of ASF virus in cell culture as false negatives test result can be generated in case of non haemadsorbing ASF virus isolates. Although, viral isolation and detection by HAD is internationally accepted diagnostic test, the result should be confirmed by other tests such as immuno-assays or PCR (Oura et al., 2002).

Previous studies revealed that non-pathogenic non-HAD ASF virus often cause sporadic viremia in infected pigs and small amounts of the virus occur in various organs of the infected pigs (Kay-Jackson et al., 2004). This makes isolation of non-pathogenic and non-HAD ASF virus more difficult than HAD virus. This could explain why probably, we were unable to isolate non HAD avirulent strains of ASF virus in this study. The non-haemadsorbing avirulent ASF virus isolates have genomic deletions unlike the HAD virus isolated (Kay-jackson et al., 2004). The low pathogenicity of some of the non-HAD isolates may be related to loss of virulence factors associated with the deleted genes which is probably associated with mutation in the gene that encode adhesion protein CD2v (Zsak et al., 2001). In Portugal and Spain, non-HAD ASF virus were isolated in many pig tissue samples during the period of attempted vaccination of pigs against ASF (Vivagio et al., 1974). Nevertheless in the Ugandan case, there were no documentation showing vaccination attempt to control or prevent ASF outbreak in the country in the previous years. The emergence of pathogenic non-HAD ASF virus isolates was probably due to natural phenomenon (mutation).

Cytopathic effect in ASF virus infected macrophages was clearly observed by the third day post infection (56 -72 h PI) in this study. The infected cells were distended and many of them were detached from the surface of the culture plates and lysed. Greig et al. (1967) pointed out that it is difficult to distinguish between the true cytopathic effect due to ASF virus and that as a result of cell degeneration caused by other factors especially in commercial cell lines. For example in vero cells, true cytopathic effect is clearer after several passages, especially between the 4th and 8th passages and it reaches its maximum between 8th and 20th passages (Greig et al., 1967). Cytopathic effect reflects the quantity of virus production and the state of adaptation of the virus in a given cell type.

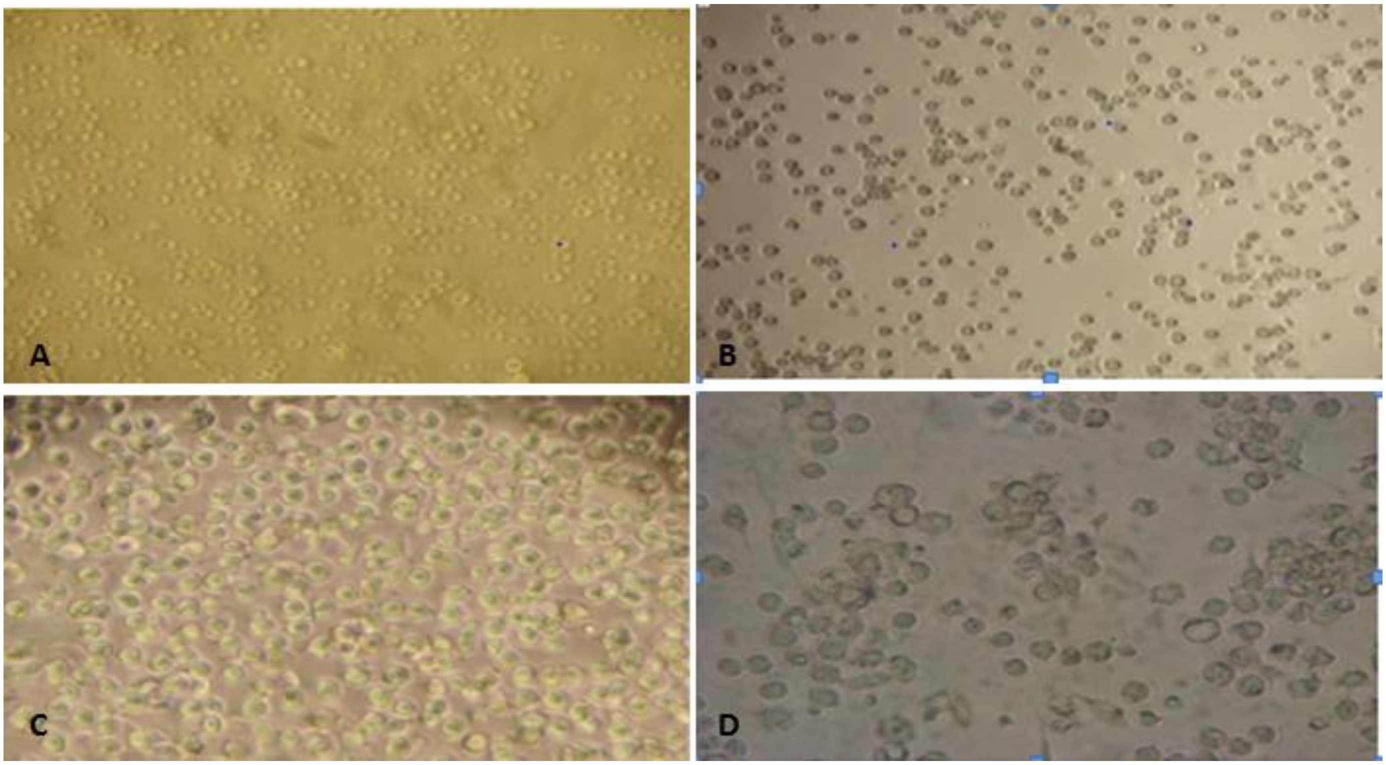

**Figure 4.** Changes observed at x10 (A and B) and x20 magnification in macrophages infected with Ugandan isolates of ASF virus. (A): non infected macrophages 72 h post infection, no changes noted in cell sizes, (B): infected macrophages 48 h PI no significant morphological change was noted, (C): infected macrophages 56 h PI, the cells were swollen and rounded. (D): Infected macrophages 72 h PI, majority of the infected cells were lysed and intact cells swollen.

Success of adapting a virus isolate to a foreign host (cell) depends on the degree of virus selection. It is usually those members of the viral population that are best suited to grow in foreign cell line that eventually become dominant in the cell culture. ASF virus generally adapts slowly to grow in pig kidney cells and cytopathic effect takes a longer time as compared to other viruses (Greig et al., 1967). Unlike what has been reported in conventional cell lines, in this study, CPE was first observed by the 56th h post inoculation in infected macrophages and by 72 h post infection majority of the cells were infected by ASF virus and had CPE (Figure 4D and B. To confirm that the observed CPE was due to ASF virus, we detected the presence of ASF viral DNA and antigens in the infected macrophages by conven-tional PCR and IPMA. The cytopathic effects of ASF virus on infected macrophages at different durations post infection were as shown in Figure 4A to D. Sanchez–Torres et al. (2003) noted that pig macrophages and monocytes *in vitro* are cells of choice for cultivation of ASF virus and field isolates of ASF virus do not replicate in conventional cell cultures. This is because ASFv naturally infects and replicates in mononuclear phagocytic cells (Sanchez–Torres et al., 2003, Malmquist and Hay, 1960). In mononuclear phagocytic cells *in vitro*, ASF virus mimics natural infection and most strains of the virus grow readily in monocytes and macrophages culture (Carolina et al., 2010). This probably could explain the early CPE observed in this study and the high infectivity of the isolates used might also contribute to the short time of the observable effect of the virus on the cell culture. The viral suspension in the sample filtrate used in this study was evaluated in terms of haemadsorbing units and the actual viral load per unit volume was not titrated. Macrophages cultures that showed early CPE (54 hours PI) probably had higher viral load than others where CPE appeared 72 h PI.

## Challenges and limitations of the study

Samples for this study were obtained from pigs reported to have died of swine haemorrhagic diseases, hence probably only virulent strains of ASF virus were isolated. Other less virulent or avirulent strains of ASF virus were not used in this study.

The study was limited to strains of ASF virus obtained from domestic pigs in Uganda, though it is known that wild swine species and soft ticks (*Ornithodoros moubata*)

which have no boundaries are the reserviour hosts and vectors of ASF virus, respectively.

## CONCLUSIONS AND RECOMMENDATIONS

Majority (95%) of the ASF virus isolates from Uganda during this study were haemadsorbing though 5% of the isolates were non haemadsorbing. Virulent African swine fever virus isolates from Uganda caused noticeable cytopathic effect in infected macrophages as early as 56 h post inoculation.

Imunoperoxidase monolayer assay was able to detect ASF viral antigens in the macrophages culture as early as 48 h post infection similar to PCR though the sensitivity of PCR is more than the former at this time. Therefore, IPMA is an appropriate option to PCR technique, which could be used to detect ASF viral antigens in cell culture especially in less established laboratories as it detects both haemadsorbing and non haemadsorbing strains of ASF virus.

We recommend that a survey should be conducted to investigate occurrence of classical swine fever and other pig haemorrhagic diseases in Uganda as only 58.8% of the pigs that had lesions suggestive of ASF had ASF viral DNA. More so, the genome of the non-haemadsorbing virulent ASF viral isolates should be sequence to see if there is nucleotide sequence variation as compared to HAD isolates. A study could also be conducted to determine the virulence factor in ASF virus isolates and explain why some non-HAD ASF virus were also pathogenic.

### Declaration of non conflict of interest

The authors declare that there is no conflict of interest in this study

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