Research Article
Volume 14:01, 2023

Journal of Veterinary Science & Technology

ISSN: 2157-7579 Open Access

Serological and Molecular Investigation of African swine fever in Central Ethiopia

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Abstract

This study was conducted between December 2016 and April 2017 in central Ethiopia to detect African Swine Fever Virus (ASFV) in export abattoir in Addis Ababa and to investigate ASF suspected outbreaks in central Ethiopia and assess associated risk factors. A study was conducted on targeted pig population slaughtered at Addis Ababa abattoirs enterprise to investigate ASFV from collected samples. 65 tissue and 104 whole blood samples were collected from fattened pig that came to Addis Ababa abattoirs enterprise from four different farms in and around Addis Ababa. The samples were submitted to national animal health diagnostic and investigation center/(NAHDIC) for the detection of the virus. The tissues were pooled together from different organs and screened by PCR using primers. The sera were separated from whole blood in the laboratory and then ASFV antibody detection was performed using the Blocking ELISA assays. Both the serology and molecular test results revealed that none were positive for ASFV. These results indicate that the pigs slaughtered at Addis Ababa abattoirs enterprise during these period were free of ASFV. But in March 2017, subsequent ASF outbreaks were notified in Adama farm with intensive farming systems. Accordingly, 34 whole blood samples were collected from the outbreak and screened by real time PCR at NAHDIC. Out of the 34 samples tested, eleven samples were positive for African swine fever virus by the OIE prescribed real time PCR. Though, the assessment for the circulation of ASFV antibodies in apparently health pigs revealed negative, the test from the outbreak confirmed the presence of African swine fever virus in Ethiopia. Thus, swine farmers and concerned authorities need to consider the situation and take appropriate action.

Keywords: Addis ababa • Food security • ELISA • PCR • Slaughtered pigs

Introduction

African swine fever (ASF) is a serious transboundary animal diseases (TAD) with the potential for rapid international spread and highly contagious and fatal hemorrhagic viral disease of domestic pigs, warthogs, wild boars, and other members of Suidae family and affects all age groups. ASF affects only species of the suidae family (both wild and domestic) of all breeds and ages, giving rise to a variety of clinical signs and lesions that vary in terms of the virus virulence, host species affected and their immunological status [1].

ASF is one of the most serious transboundary swine diseases because of its high lethality for pigs, its crippling socio-economic consequences, its propensity for rapid and unanticipated international spread, and causes adverse effects on both regional and international trade, food security at household and commercial levels due to the direct loss of pigs, and affecting the livelihoods of pig keepers in endemic countries of Africa, Since there is no vaccine currently available, slaughter and quarantine are the only methods of control.

Many factors, including the complex epidemiology, with the presence of natural ASF reservoirs, the carrier animals, its potential for endemicity and the resistance of the virus in the environment represent significant challenges for ASF control. ASF is difficult to control because (i) no vaccine is available at the moment, (ii) African swine fever virus (ASFV) infection does not elicit the production of neutralizing antibodies and (iii) the soft ticks Ornithodoros spp which act as a vector of the disease are widely distributed and difficult to control. The severity of ASF ranges from sub-acute to highly lethal acute disease depending on the doses and viral isolate involved European wild boar (Sus scrofa) and feral pigs are very susceptible to the disease and exhibit similar clinical signs and lethality to domestic pigs. By contrast, infected wild African Suidae develops subclinical and asymptomatic long-term persistent infections, acting as virus reservoirs [2-4].

When introduced into disease-free regions or domestic pig population, per acute and acute forms of the disease are predominant. These forms of ASF result in high mortality rates of up to 95-100% within 4–9 days post-infection. However, after several years of ASFV presence, sub-acute or chronic forms may be present, and mortality rates decline over time. In infections with low virulent ASFV isolates, the clinical manifestations of the disease are more variable and difficult to recognize in the field. The infection can persist for several months without obvious clinical signs in the infected animals. Sub-clinically infected, chronically infected, or surviving pigs are likely to play an important role in the epidemiology of the disease, for example resulting in disease persistence in endemic areas or in sporadic outbreaks of ASF into previously ASFV free zones.

The risk of new introductions or reintroductions of the virus in ASF-free areas is high, taking into account the complex epidemiological situation of ASF in Africa. In eastern and southern African countries, ASFV has been maintained, for almost a century, in an ancient sylvatic cycle involving soft ticks (Ornithodorus genus) and asymptomatic wild African pigs, mainly warthogs (Phacochoerus spp), which can act as potential long term carriers allowing the virus to spill over into domestic species when the two interact. Two additional cycles have been described in endemic areas, namely a domestic pig/tick cycle, without warthog involvement, and a domestic pig/pig cycle in which the virus persists in domestic pigs in absence of vertebrate or invertebrate hosts when proper disinfection measures are not carried out [5,6].

The disease is caused by ASFV the only member of the Asfaviridae family, which is an enveloped icosahedral arbovirus of the genus Asfivirus. ASFV has a number of strategies to evade the host's defense systems, including innate

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Received: 16 January 2023, Manuscript No. jvst-23-87171; **Editor Assigned:** 18 January 2023, PreQC No. P-87171; **Reviewed:** 01 February 2023, QC No. Q-87171; **Revised:** 07 February 2023, Manuscript No. R-87171; **Published:** 15 February 2023, DOI:10.37421/2157-7579.2023.14.163

and intrinsic immune mechanisms such as type I IFN responses, apoptosis, inflammation and activation of host immuno modulatory gene expression. ASF virus replicates primarily in cells of the reticulo-endothelial system and consequently blood, lymph node, spleen, liver and tonsil are the preferred specimens for laboratory examination.

Even though small scale commercial pig farming is emerging in Ethiopia, there is little information on prevalence and epidemiology of ASF in the country. There is only a single ASF outbreak report from Ethiopia in which a new genotype of ASF virus was reported. Therefore, this study was initiated with the objectives of detecting ASFV in Addis Ababa abattoirs and investigates ASF suspected outbreaks in central Ethiopia. The specific objectives of the study were:

- To detect ASFV in in apparently health pigs slaughtered at Addis Ababa abattoir
- To investigate ASF suspected outbreaks in central Ethiopia and assess associated risk factors
- To determine the prevalence of ASFV in slaughter pigs in Addis Ababa abattoirs enterprise (AAAE).

Materials and Methods

Study area

The study was conducted at AAAE. Addis Ababa is capital city of Ethiopia, situated at 11°1'48"N and 39°37'59.83"E, at an altitude of 2000 m a.s.l. up to 3000 m a.s.l the city has a subtropical highland climate. The mean annual rainfall is 1800 mm with a bimodal pattern, whilst the daytime mean annual minimum and maximum temperatures are 14°C and 21°C respectively.

Additional investigation was conducted in Adama where the outbreak of the disease occurred in March 2017. Adama is located at 8° N latitude and 39° E longitude about 99 km Southeast of Addis Ababa. It is placed at an altitude ranging from 1600 to 1700 meters above sea level. The area entertains an average annual rainfall ranging from 600 to 1150 mm which is inconsistent in nature. More than 67% of the mean annual rainfall showers in June, July, August and September. Some additional rains (about 23%) taken place during the remaining dry months with sometimes mean monthly rainfall of being zero. The minimum and maximum daily temperatures of the area are 12 and 33°C, respectively.

Study animals

The animals studied were swine population presented for slaughtering at the Addis Ababa Abattoirs Enterprise from different farms, which include Bishoftu, Addis Ababa (Akaki and keraneo) and Tatek farms. AAAE is located at Nifas silk Lafto kifla ketama (local name Kera). The age of swine slaughtered was estimated according to their teeth. It was impossible to get the exact value of the age for each slaughtered swine population during the study period except sex, but all were adults. The study animals were managed under intensive farming system in the selected farm of the study area [7].

Study design

A cross sectional study was conducted on targeted pig population slaughtered at AAAE to investigate ASFV from whole blood and tissue samples which included liver, lungs, heart, kidneys, spleen and mesentery. A systematic random sampling procedure was employed to carry out this study. The sample size for this study was determined based on the pig population slaughtered at AAAE per week. Considering the equipment in the molecular and viral serology laboratory were expensive.

Sample collection and analysis

Sampling was undertaken from December 10/12/2016 to April 3/4/2017 during the dry season. Tissue and whole blood samples were collected from domestic pigs in districts with suspected ASFV cases based on clinical signs and post-mortem lesions. The samples were put in to bottle containing viral transport media (VTM) and transported to NAHDIC laboratory for further investigations of the virus. DNA extraction and PCR was performed at Molecular laboratory at NAHDIC. Additionally, a whole blood sample was collected in March, 2017 from an outbreak reported in Adama town for the diagnosis of ASFV.

Detection of ASFV from whole blood and tissue samples

DNA extraction: For DNA extraction pooled tissue samples were homogenized in VTM with help of sterilized pestle and mortal. Each homogenized specimen was transferred to a 1.5 ml labeled microbial centrifuge tube. 20 µl QIAGEN protease K was added and centrifuged. Lysis buffer was added to the sample and mixed by pulse vortex until completely dissolved and incubated for ten minutes (10 minutes). Then briefly centrifuge the 1.5 ml micro centrifuge and a binding step with 70% ethanol was performed and 500 µl buffer AW1 and AW2 was added to the QIAamp mini spin column and centrifuged at full speed 20,000 rpm for 3 minute. The Elusion step was followed by Adding 200 µl AE and then incubated at room temperature for 1minute and centrifuged at 8000 rpm for 1minute. The extracted DNA was stored at -20°C until further use. Whole Blood was collected in vacutainer tube containing inhibitors for detecting the disease agent [8].

Real time polymerase chain reaction: Real time PCR was conducted after preparing the master mix. Preparation of the master mix was done according the manufacturer's recommendations. The master mix contains dNTPs, Primer Forward & Primer Reverse, Probe, Rnaseinhabitor, Rnase free water and Tag polymerase. For amplification of target gene, the Thermal Cycler (Applied bio system 7500 Fast real time PCR system) was programmed as follows: Initially the temperature was set at 95 OC for 15 min for activation of the Tag polymerase enzyme. The denaturation, annealing and extension temperature was set at 94OC for 10 sec, 56 OC for 30 sec and 72 OC for 25 sec respectively. This was repeated for 40 cycles. Then result was read from the software.

Blocking ELISA: The blood samples collected from apparently healthy pigs at AAAE were checked for the presence of ASFV antibodies using a blocking ELISA. The blood samples for this purpose were collected from the ear vein in plain tubes and were put upside position on the test tube ruck. Then the serum was collected manually after 24hr and stored at -20ºC till used in the ELISA assay.

Detection of serum antibody in the swine was done using blocking ELISA according to the procedure described by the kit manufacturer. Briefly, 50 µl of supplied diluents was added to each well of the micro-plate coated with a purified protein extract from the virus (vp72), which is the major structural protein from the ASFV and the most antigenic one. Then, 50µl positive and negative control sera were added to two wells (i.e. A1 and B1 for positive and A2 and B2 for negative). After this 50µl serum samples were added to the remaining wells of the plate. Then the plate was sealed with plate sealer and incubated for one hour at 37OC. After this all wells were emptied and washed 4 times with washing buffer supplied with the kit. Then, 100µl of conjugate, specific to ASFV, was added to each well of the plate and plate was sealed and incubated at 37OC for 30 minutes. After this, it was washed 5 times and 100 µl of substrate was added to each well. Then, the plate was kept for 15min at room temperature. Finally, 100 µl stop solution was added to each well and read the optical density (OD) at 450nm with 5min after the addition of stop solution.

The test was considered to be valid when the OD of the Negative control (NC) is, higher than the OD of Positive control (PC) and NC/PC has to be greater than or equal to 4. The cut off value was calculated according the kit as follows:

Positive cutoff = NC-[(NC-PC) × 0.5] Where: NC=OD of Negative control serum

Negative cutoff = NC-[(NC-PC) × 0.4] PC =OD of Positive control sera

For calculating the blocking % (x%) of a sample:

$$X\% = \frac{\text{NC} - \text{sample OD}}{\text{NC} - \text{PC}} \text{x } 100$$

Results were interpreted as positive, negative and doubtful when blocking percentage was ≥50%, ≥40% and between both values respectively.

Data analysis

All data were entered in to micro-soft excel spread sheet and coded appropriately. Then these data were distributed normally and the differences linked to age and sexes were analyzed by simple descriptive statistics using a statistical software program (SPSS).

Results

Detection of ASFV from tissue samples of slaughtered pig by PCR

During the study period, a total of 169 domestic pigs were brought by butchers and slaughtered at Addis Ababa Abattoirs Enterprise which were originated from different farms and revealed no clinical signs of ASF. Further post-mortem examination of the carcasses showed no lesions indicative of disease.

65 tissue samples were collected from the slaughtered pigs which came from four different farms. The tissue samples were pooled together from different organ and screened by PCR using primers. Out of 12 pooled tissue samples screened by PCR, none of the samples revealed positive result for ASF. This result indicate that the animals slaughtered at AAAE during these period were disease free (Table 1).

Outbreak investigation of ASFV

In March 2017, subsequent ASF outbreaks were notified from swine farms in Adama with intensive farming systems where pigs were confined and had no potential interaction with wildlife. Affected animals presented an acute form of ASF with anorexia, cyanotic skin, grunting and depression prior to death. Of the total 34 whole blood samples collected from farms where outbreak was reported, 11 samples were ASFV positive by the OIE-prescribed real-time PCR (Table 2).

Detection of ASF antibodies by using the blocking ELISA

Among the 104 blood samples collected from pigs that came from different farms in and around Addis Ababa and slaughtered at AAAE, none of the samples were positive for ASFV antibodies through the blocking ELISA technique.

Discussion

The outbreak investigation result in the present study indicated for the presence of ASF in Ethiopia, considering that the sample sites are the major pig rearing region, as compared with other region of Ethiopia. A similar result was also reported in Ethiopia from a study conducted from 2011-2014.

Pigs slaughtered in the AAAE represent pig populations from virtually the central Ethiopia; therefore, this study depicts a representative status of ASF in those districts. The tissue samples and whole blood samples which were collected from the slaughtered pigs and analyzed at NAHDIC for the presence of ASF revealed that pigs slaughtered during this period were disease free. This could be due to the careful selection of the healthy and good conditioned pigs from those farms in and around Addis Ababa by the butchers. As to the information obtained from the people involved in this business, they select only pigs that have good body condition and shiny hair coat which have no any sign of abnormality like nasal discharge, depression, and any bleeding [9].

This study indicated that there was outbreak of ASFV at Adama in March 2017; which was notified in farms with intensive farming systems where pigs were confined and had no potential interaction with wildlife. During the study period many tourists came to the town to visit Aba-Gada hall and Sodare resort; these tourists could be the predisposing factor to the outbreak because the virus might be introduced into the city through tourist's clothes, shoes and equipment. Achenbach and colleagues (2016) reported the presence of ASFV in Ethiopia which was reported in January 11, 2016. This study confirmed that the hemorrhagic disease with high mortality rate to pigs in central Ethiopia was ASF after performing real-time PCR using diagnostic primers PPA1/PPA2. Confirmation of ASF using PPA1 and PPA2 primers have been used elsewhere.

Table 1. Surveillance of ASFV infection in domestic pig on tissue samples collected from pig slaughtered at AAAE from December, 2016-April, 2017.

No.	Origin	Age Sex			No. of Tissue Sample	PCR Result
		Young M	Adult F	Old		
1	Bishoftu	7 19	15 5	2	24	NEG
2	AA(keraneo)	8 10	5 6	3	16	NEG
3	AA(Akaki)	4 12	12 4	0	16	NEG
4	Tatek	3 9	6 0	0	9	NEG
	Total	18 50	42 15	5	65	NEG

Table 2. In March, 2017 whole blood were collected from domestic pigs in Adama for diagnosis of outbreak.

Origin	Age		Sex		Sample	PCR result	
	Young	Adult	M	F		POS	NEG
Adama	20	14	27	7	Whole Blood	11	23

In ideal situations diagnosis of ASF should be carried out through a combination of tests including the detection of viral genome by real-time PCR and the detection of viral antibody by blocking ELISA. PCR is highly specific and sensitive. PCR has demonstrated its superior diagnostic capacity in ASF detection in clinical disease conditions and in sub-clinical manifestations; therefore, this technique should be employed in diagnostic laboratories.

When introduced into a region or a domestic pig population, ASF is typically associated with high mortality rates and a rapid spread of outbreaks. However, several studies have demonstrated that in areas where ASF becomes endemic, increased numbers of subacute, chronic and subclinical infections also occur, and that mortality rates decline over time. In such situations, the clinical manifestations of this disease are more variable and difficult to recognize in the field. The infection can persist for several months with no particular symptoms in the infected animals, other than stunting or emaciation, or may even mimic certain other illnesses.

The persistence of the virus in organs such as the respiratory tract implies a risk factor for virus transmission to other animals, which thus could contribute to the spread and maintenance of the disease. This finding has been reported in other experimental studies that have identified tissues as a source of virus during persistent infections with moderately virulent isolates.

Some studies have reported that in sub-acute infections using ASFV isolates of moderate virulence, viraemic surviving pigs were able to shed virus from their oropharynx for at least 70 days.

There is currently no data regarding the presence of ASF in other boarder countries such as Sudan, South Sudan, Eritrea, Djibouti and Somalia. While many of these neighboring countries, due to religious preferences, have minimal domestic swine, the sylvatic cycle between warthogs and ticks remains a possibility and thus should be investigated [10].

Though the molecular detection from tissues samples and serology from whole blood samples of slaughtered pigs revealed negative results in the present study, the outbreak investigation result highlighted for the potential circulation of ASFV in the pig populations of the country.

Conclusion and Recommendations

The presence of the ASFV in Ethiopia was confirmed after the whole blood samples collected from outbreak in pig farms in Adama were screened by OIE-prescribed real-time PCR. However, the molecular and serology test results from apparently healthy pigs slaughtered at AAAE were negative for all the samples tested. Movement of the tourists across the border had a possibility to spread the disease. An indirect infection through a vector, object or person from wild boar to domestic pigs depends on good biosecurity. The inability of ASFV to induce neutralizing antibodies has hampered the prevention and control of the disease by vaccination and to date there is no vaccine for ASFV. In the absence of effective vaccines, control based on rapid laboratory diagnosis and the enforcement of strict sanitary measures is considered important. The potential presence of ASF in Ethiopia could pose a significant economic impact in swine production because ASF is typically associated with high mortality rates and a rapid spread of outbreaks.

Therefore, from the finding of this study the following recommendations are forwarded:

- The confirmation for the presence of ASF from the outbreak is an alarming signal for the country. Thus, swine farmers and concerned authorities need to consider the situation seriously and take appropriate actions.
- Farmers of the infected area should be encouraged to enhance biosecurity practices. Sharing of equipment between the pig farms should be discouraged
- Visitors should be discouraged to enter the pig farm, especially the commercial ones.
- Appropriate hygiene measures have to be applied by all persons entering into contact with pigs.
- Pig farmers and the operators of the pig sector should be made aware of ASF

Acknowledgements

First of all, I would like to praise the GOD by the name of Jesus Christ because of his almighty acts he has done for me in my life and especially at the time of preparing this research paper. I am really happy to express my heart appreciation and thanks to my advisors Habtamu Taddele, Delesa Damena and Belayneh Getachew for their heart full intellectual advice, comments, suggestion and support during editing this research paper by scarifying much time on it. My appreciations also go to NAHDIC for permitting me by fulfilling necessary materials and reagents during the study period. And special thanks also go to laboratory technicians who are heartily sharing their experience during my laboratory work. And also I am so grateful to express my greatest respect to my family especially Aster and Alemeyehu for their financial support and moral encouragement. I want to convey my deepest thanks to Abdi Aliy who assisted me at the time of sample collection during field work and to all my friends because of their special support in different ways.

Declaration

I declare that:

- This thesis presents the work carried out by myself and does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university, and
- To the best of my understanding, it does not contain any materials previously published or written by another person except where the reference is made in the text; all substantive contributions by others to the work presented including jointly authored publications, is clearly acknowledged.

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How to cite this article: Gemechu, Abenezer. "Serological and Molecular Investigation of African swine fever in Central Ethiopia." *J Vet Sci Techno* 14 (2023): 163.