EPIDEMIOLOGY OF AFRICAN SWINE FEVER IN NIGERIA

by

Folorunso Oludayo FASINA

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy (Zoology)
in the
Department of Zoology and Entomology
Faculty of Natural and Agricultural Sciences
University of Pretoria

Supervisor: Prof. A. D. S. Bastos

Co Supervisor: Dr. C. Gallardo

Pretoria, October, 2012

ACKNOWLEDGEMENTS

I wish to express my unreserved gratitude and appreciation to the following:

Prof. A. D. S. Bastos for her confidence in me, unhindered advice, scientific guidance, and the unwavering moral and financial support throughout the duration of the project,
Dr. C. Gallardo for her effort in training, making available laboratory space in Spain and guidance that contributed to the successful completion of this work,
Dr. Livio Heath, and staff for collaborating fully for the success of the project,
The Dean, Faculty of Veterinary Science, University of Pretoria, Professor Gerry Swan and Head of Department, Professor Pete Irons and staff of the Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria for moral support,
Prof. K. P. Pettey, Prof. G. Bath, Dr. R. Leask, Dr. D. Coetzee, Lana Botha and Mrs. E. Orsmond, my able office colleagues in the Section under the Department;
Dr. Peter Smith and his family,
The staff of the Department of Zoology and Entomology, especially Stokana Mahapa and the MEZ Group for regular assistance,
My friends Matthew Adamu, Akin Jenkins, Lazarus David, Oyinlola Olaokun, Fikemi Iji, Okechukwu Ndumnego, Mai Hassan, M. B. Umar and others too numerous to mention for their supports,
Mrs. Ingrid Booysen, Department of Geography, Geoinformatics and Meteorology, University of Pretoria, for the maps, and Ms Idette Noomé, Department of English, University of Pretoria for certain English revisions,
Pastor Robert Greenland and friends at the Pretoria North Methodist Church, all too numerous to mention for their encouragement,
All who contributed in one way or the other to the successful completion of this work,
My parents, Mr. J. B. Fasina and Mrs. M. A. K. Fasina for believing in me from childhood, and my siblings,
My sons, Temitope and Tolulope, my daughter, Temitayo and my beloved wife Modupe who supported me fully during the study period.
My appreciation also goes to the following for immense financial support:

- Transboundary Animal Disease Programme, ARC-Onderstepoort Veterinary Institute, Pretoria, South Africa for provision of viruses, laboratory space and facilitation of work,
- International Livestock Research Institute, Kenya; Centro de Investigación en Sanidad Animal, CISA-INIA, Madrid, Spain and European Union ASF Risk Project for training, provision of viruses and laboratory space.
- MEZ Group, Department of Zoology and Entomology, University of Pretoria, and
- Agricultural Research Council of Nigeria, Abuja for partial funding of this project and the National Veterinary Research Institute, Vom, Nigeria.

Ultimately, I thank the Almighty God for continued opportunity to successfully conclude this research.

"To God be the glory great things he hath done"

-Frances Jane Cosby (1820-1915)

DECLARATION

I, FASINA, Folorunso Oludayo declare that the thesis, which I hereby submitted for the degree of PhD (Zoology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature: ……………………..

Date: ……………………….…….

DISCLAIMER

This thesis consists of a series of chapters that have been prepared as stand-alone manuscripts for publication in different scientific journals. Consequently, unavoidable overlaps and /or repetitions may occur and the reference style and format may differ between chapters.

Signature: ……………………..

Date: …………………….…….

Summary

African swine fever (ASF) is a highly contagious transboundary viral infection of domestic pigs that has serious socio-economic implications on people's livelihood, international trade and food security. It is still a major limitation to profitable pig production and presently, it is threatening the pig industry internationally with current outbreaks in the Russian federation and the Caucasus. Since 1996, the disease has made major incursions into the West African sub-region.

In this study, a combination of classical epidemiologic (statistical), economic, laboratory (serological, virological and molecular) and evidence-based tools were used to determine the prevalence of ASF in Nigeria, map the temporal situation of the virus, estimate the economic implications of infection with justification of alternative control (biosecurity), compare and contrast the virulence genes (*Thymidine kinase, TK*) and finally evaluate the effectiveness of ethnoveterinary preparations used in the management of ASF.

Nine percent (9%) of serum samples and 48% of tissue samples tested were positive for ASF virus antibody and genome, respectively. Areas with high pig-related activities (marketing, consumption and farming) have higher prevalences compared with areas with less pig activities. Farm-gate buyers, marketing systems and transport of untested pigs within the country appeared to assist with the circulation of the virus. Using the financial model of partial budgeting and benefit-cost analysis, ASF outbreak in a 122-sow unit may lead to a loss of up to ZAR7,475,867.27 (US$910,836.70) in a single year while the implementation of biosecurity and its effective monitoring can prevent these losses with certain other social benefits and give a benefit-cost ratio of approximately 29 in return, but the cost of its implementation may result in a 9.70% less annual profit.

Since the identification of factors that supports infection on pig farms in the sub-region remains the key component in the development of a risk-based approach to control the

disease, most plausible risk factors and biosecurity measures previously identified were analysed in this study with a univariable/multivariable conditional logistic regression analytic models. Presence of an abattoir in a pig farming community (OR = 8.20; $P < 0.001$) and the presence of an infected pig farm in the neighbourhood (OR = 3.26; P = 0.02) were significant risk factors. There was a marginally significant negative association (protective) between risk of ASF infection and sharing farm tools and equipment (OR = 0.35; P = 0.05). For the biosecurity measures evaluated, food and water control (OR = 0.14; $P < 0.001$), separation/isolation of sick pigs (OR = 0.14; P = 0.004) and washing and disinfection of farm equipment and tools (OR = 0.27; P = 0.02) were negatively associated (protective) with ASF infection. Consultation and visits by veterinarian/paraveterinarians when animals were sick (OR = 8.11; P = 0.002), and pest and rodent control were positively associated with ASF infection of Nigerian farms (OR = 4.94; P = 0.002). The leaf, root and stem portions of *Ancistrocladus korupensis* possessed some chemical compounds with antiviral potentials and extracts and fractions from the plant showed very good antiviral (virucidal) activities *in-vitro* against ASF virus (NIG/99). It also has certain cytotoxic principles and narrow therapeutic index. Further studies on the maximization of the ethnoveterinary potentials of the plant *in-vivo* and *in-vitro* while reducing its cytotoxic potentials will be needed.

Using molecular characterization, similar unresolved topologies were observed within the European, South America, Caribbean and West African (ESAC-WA) genotype and the mean character distances on the coded data set revealed least possible loss of information that would have otherwise been ignored in pairwise- or complete- deletion distance analysis. The size of the coding ORF for the TK protein varies between isolates but the majority of isolates code for a protein of 196 amino acids. These isolates comprise of viruses from Europe, West, Central and Southern Africa. A smaller *TK* gene product of 185 and 194 amino acids, caused due to a frameshift mutation at nucleotide position 561 in many of the East African isolates

resulting in stop codons immediately thereafter or further downstream (nucleotide position 571 in Malawi 3). Despite the smaller TK protein product size, certain nonsense insertions of differing length were responsible for some considerably larger *TK*-PCR products.

This TK protein heterogeneity is unexpected in an enzyme with such an important function and these size differences may have an effect on virulence. It is concluded that strains from southern Africa may have a shared evolutionary history with strains of the ESAC-WA genotype but may differ from the evolutionary lineage from East Africa. It is also suggested that a link exist between the sylvatic cycle, domestic tick cycle and the truncated *TK* products.

Finally, putting in place a comprehensive routine surveillance and testing system to rapidly eliminate all pigs in infected farms, reorganization of the market and transportation systems for pigs, implementation of carefully planned on-farm biosecurity protocols, and giving consideration to the option of compensation to encourage reporting of outbreaks will possibly achieve a significant reduction in high ASF prevalence in Nigeria.

It will be desirable to eliminate certain risky farm-related practices and behaviours (e.g the removal of all pig abattoir from within the pig communities, isolation of infected neighbourhood farm) while entrenching farm-level biosecurity as these appear to be the key to controlling ASF within the subregion. In conclusion, the outcomes of this research can be used to plan long-term strategies for countries like Nigeria, and assist the ASF unaffected countries that are at risk of infection to organize and secure their animal (pig) resources, so that Africa can be free from the significant effects of ASF and explore options of international markets.

Scientific publications arising from this thesis

Manuscript published from the thesis:

1. **Fasina FO**, Shamaki D, Makinde AA, Lombin LH, Lazarus DD, Rufai SA, Adamu SS, Agom D, Pelayo V, Soler A, Simon A, Adedeji AJ, Yakubu MB, Mantip S, Benshak AJ, Okeke I, Anagor P, Mandeng DC, Akanbi BO, Ajibade AA, Faramade I, Kazeem MM, Enurah LU, Bishop R, Anchuelo R, Martin JH, Gallardo C, 2010. Surveillance for African swine fever in Nigeria, 2006-2009. ***Transboundary and Emerging Diseases***, 57: 244-253.
2. **Fasina FO**, Lazarus DD, Spencer BT, Makinde AA, Bastos ADS, 2012. Cost implications of African swine fever in smallholder farrow-to-finish units: Economic benefit of disease prevention through biosecurity. ***Transboundary and Emerging Diseases***, 59: 244-255.
3. **Fasina FO**, Agbaje M, Ajani FL, Talabi OA, Lazarus DD, Gallardo C, Thompson PN, Bastos ADS, 2012. Risk factors for farm-level African swine fever infection in major pig-producing areas in Nigeria, 1997-2011. ***Preventive Veterinary Medicine***, 107: 65-75, doi: 10.1016/j.prevetmed.2012.05.011

Manuscript under development/submitted for publications:

1. **Fasina FO**, Olaokun O, Oladipo O, Fasina MM, Makinde AA, Heath L, Bastos ADS. Phytochemical analysis and in-vitro anti-African swine fever virus activity of extracts and fractions of *Ancistrocladus korunpensis,* Thomas and Gereau (Ancistrocladaceae). Submitted to ***BMC Veterinary Research***.
2. **Fasina FO**, Edrich JL, Van Heerden J, Heath L, Gallardo C, Vosloo W, Gerber LJ, Bastos ADS. Length and sequence heterogeneity of the *Asfivirus thymidine kinase* gene. Prepared for ***Virology Journal***.

Conference Proceedings and Posters arising from or associated with the thesis:

1. **Fasina FO,** Shamaki D, Makinde AA, Lombin LH, ASF PITT, 2009. Field surveillance and laboratory diagnoses of African swine fever in Nigeria. *International Symposium on Sustainable Improvement of Animal Production and Health*. 8-11 June 2009, Vienna Austria, IAEA-CN-174-151. Pp300-302. Also available at: http://www-naweb.iaea.org/nafa/aph/BookOfExtendedSynopses.pdf .

2. Akanbi O, **Fasina F,** Teifke J, Okewole P, Makinde A, Shamaki D, 2010. ASF: Comparison of immunohistochemistry detection, polymerase chain reaction and ASF virus isolation from farm sites, slaughter slabs and abattoir tissues in Nigeria. 28th ***Meeting of the European Society of Veterinary Pathology and the European College of Veterinary Pathologists***, 8-11 September, 2010, Belgrade, Serbia. Poster (P43) Poster seminar Tour 3A. Infectious Diseases.
3. Gallardo C, Soler A, Simón A, Martín E, Martín R, Pelayo V, Okoth E, Bishop R, Sánchez MA, de Mia G, **Fasina FO**, Sánchez-Vizcaíno JM, Arias M, 2010. African swine fever Threat: Evaluating diagnostic tools with ASFV circulating strains. ***Annual Meeting of National African swine fever and Classical swine fever Laboratories***, 18 May, 2010, Pulawy, Poland, Book of Abstracts.
4. **Fasina FO**, Agbaje M, Ajani FL, Thompson PN, Bastos ADS, 2012. Risk factors for African swine fever and the effect of biosecurity. 13th Conference of the ***International Society for Veterinary Epidemiology and Economics*** (ISVEE 13), 20-24 August 2012, Maastricht, the Netherlands and Ghent, Belgium. Poster.
5. **Fasina FO**, Agbaje M, Ajani FL, Thompson PN, Bastos ADS, 2012. Risk factors for African swine fever and the effect of biosecurity. ***South African Society for Veterinary Epidemiology and Economics*** (SASVEPM) Congress, 1-3 August 2012, Pretoria, South Africa. Oral.
6. **Fasina FO**, Lazarus DD, Spencer BT. (2012). The feasibility of biosecurity for small-scale pig farms: A cost-benefit analysis. South African Veterinary Association Vet and Paravet Congress, 4-6 August 2012, Pretoria, South Africa. Oral.

Table of Contents

Page

ACKNOWLEDGEMENTS -- ii

SUMMARY -- iv

REFERRED ARTICLES AND CONFERENCE PROCEEDINGS -------------- viii

LIST OF FIGURES --- xv

LIST OF TABLES --- xvii

LIST OF ABBREVIATIONS -- xx

CHAPTER ONE: GENERAL INTRODUCTION

1.1 AFRICAN SWINE FEVER EPIDEMIOLOGY -- 1

1.2 AFRICAN SWINE FEVER VIRUS CHARACTERISTICS ---------------------------- 4

1.3 GLOBAL SPREAD OF AFRICAN SWINE FEVER ------------------------------------ 7

1.4 SOURCES OF INFECTIONS AND MANAGEMENT STRATEGIES -------------- 13

1.5 ROLE OF PIGS AND ECONOMIC LOSSES ASSOCIATED WITH ASF --------- 14

1.6 AIMS AND OBJECTIVES -- 15

1.6.1 Aims -- 15

1.6.2 Objectives -- 16

CHAPTER TWO: RATIONALE AND APPROACH TO THE STUDY

2.1 ASF EPIDEMIOLOGY IN WEST AFRICA --- 17

2.2 POTENTIAL RISK FACTORS FOR AFRICAN SWINE FEVER ------------------- 23

2.3 ASF AND BIOSECURITY IN PIG FARMS --- 24

2.4 ECONOMIC COSTS OF AFRICAN SWINE FEVER --------------------------------- 26

2.5 DETERMINATION OF VIRULENCE OF ASF -- 27

CHAPTER THREE: SURVEILLANCE FOR AFRICAN SWINE FEVER IN NIGERIA, 2006-2009

Page

3.0.1 SUMMARY -- 31
3.1 INTRODUCTION -- 32
3.2 MATERIALS AND METHODS -- 34
3.2.1 Location --- 34
3.2.2 Sample collection -- 35
3.2.3 ASF antibody detection --- 40
3.2.3.1 Indirect enzyme-linked immunosorbent assay OIE –ELISA ---------------------------- 40
3.2.3.2 Immunobloting -- 41
3.2.4 ASF virological detection -- 41
3.2.4.1 Nucleic acid extraction and genomic DNA amplification ------------------------------ 41
3.2.4.2 Virus isolation --- 42
3.2.5 Statistical analysis --- 43
3.3 RESULTS --- 43
3.3.1 Serology -- 43
3.3.2 DNA Demonstration and Virus Isolation -- 44
3.4 DISCUSSIONS --- 47
3.5 REFERENCES -- 51

CHAPTER FOUR: COST IMPLICATIONS OF AFRICAN SWINE FEVER IN SMALLHOLDER FARROW-TO-FINISH UNITS: ECONOMIC BENEFITS OF DISEASE PREVENTION THROUGH BIOSECURITY

4.0.1 SUMMARY -- 56
4.1 INTRODUCTION -- 57
4.2 MATERIALS AND METHODS -- 59
4.2.1 Choice of farms -- 59
4.2.2 Identification of risk factors associated with African swine fever virus introduction -- 60
4.2.3 Parameters and assumptions --- 62

Page

4.2.4 Sensitivity analysis -- 65
4.3 RESULTS -- 66
4.3.1 Profitability of the 122-sow farrow-to-finish farm and losses associated with potential ASF infection -- 66
4.3.2 Benefit of biosecurity against African swine fever compared with no-biosecurity using benefit-cost analysis (BCA) --------------------------------- 70
4.3.3 Sensitivity analysis -- 71
4.4 DISCUSSIONS --- 73
4.5 REFERENCES --- 76

CHAPTER FIVE: RISK FACTORS FOR FARM-LEVEL AFRICAN SWINE FEVER INFECTION IN MAJOR PIG-PRODUCING AREAS IN NIGERIA, 1997-2011

5.0.1 ABSTRACT -- 81
5.1 INTRODUCTION --- 83
5.2 MATERIALS AND METHODS -- 85
5.2.1 Study locations and mapping of the spread of ASF, 1997-2009 ------------------------ 85
5.2.2 Case farm definition --- 86
5.2.3 Control farm description --- 87
5.2.4 Data collection: the questionnaire --- 88
5.2.5 Statistical analysis -- 88
5.3 RESULTS -- 90
5.3.1 Spatial and temporal patterns -- 90
5.3.2 Case-control study -- 90
5.4 DISCUSSIONS --- 98
5.5 CONCLUSIONS -- 105
5.6 REFERENCES -- 106

CHAPTER SIX: PHYTOCHEMICAL ANALYSIS AND IN-VITRO ANTI-AFRICAN SWINE FEVER VIRUS ACTIVITY OF EXTRACTS AND FRACTIONS OF *ANCISTROCLADUS KORUNPENSIS,* THOMAS AND GEREAU (ANCISTROCLADACEAE)

Page

6.0.1 ABSTRACT ---------- 113
6.1 BACKGROUND ---------- 114
6.2 METHODS ---------- 116
6.2.1 Pre-screening of ethnoveterinary products used for ASF management, plant collection and identification ---------- 116
6.2.2 Phytochemical screening of leaves, stem and roots of *Ancistrocladus korupensis* ---------- 116
6.2.3 Determination of chemical compounds from *A. korupensis* ---------- 117
6.2.4 Extractions and fractions from *A. korupensis* ---------- 117
6.2.5 Phytochemical analysis on silica gel ---------- 118
6.2.6 African swine fever virus and the primary bone marrow culture ---------- 119
6.2.7 Cell viability and cytotoxicity assays ---------- 119
6.2.8 Antiviral assays of extract of *A. korupensis* and its fractions on African swine fever ---------- 120
6.2.8.1 Cell culture assay system ---------- 120
6.2.8.2 PCR and real-time PCR ---------- 121
6.2.8.3 Re-infectivity Assay ---------- 122
6.3 RESULTS ---------- 122
6.3.1 Phytochemical screening of leaves, stem and roots of *Ancistrocladus korupensis* ---------- 122
6.3.2 Determination of chemical compounds from *A. korupensis* ---------- 123
6.3.3 Phytochemical constituents expressed on silica gel ---------- 124
6.3.4 Cell viability and cytotoxicity assays ---------- 125
6.3.5 Antiviral assay of extract of *A. korupensis* and its fractions on African swine fever virus ---------- 126
6.3.6 PCR and real-time PCR (QPCR) assays ---------- 127
6.3.7 Re-infectivity Assay of extract of *A. korupensis* ---------- 128

Page
6.4 DISCUSSIONS -- 128
6.5 CONCLUSIONS -- 130
6.6 REFERENCES -- 132

CHAPTER SEVEN: LENGTH AND SEQUENCE HETEROGENEITY OF THE *ASFIVIRUS THYMIDINE KINASE* GENE

7.0.1 ABSTRACT -- 139
7.1 INTRODUCTION -- 140
7.2 MATERIALS AND METHODS -- 141
7.2.1 Selection of candidate viruses -- 141
7.2.2 DNA extraction, amplification and purification -- 142
7.2.3 Sequence analysis --- 143
7.3 RESULTS --- 143
7.4 DISCUSSIONS -- 149
7.5 REFERENCES -- 151

CHAPTER EIGHT: GENERAL DISCUSSION AND CONCLUSIONS ----- 155

Page
REFERENCES -- 161

LIST OF FIGURES

Page

Fig. 1.1 Representative pictures of wild and domestic suids involved in the epidemiology of ASF in Africa -- 3

Fig. 1.2 Electon micrographs and schematic representation of the lipid membranes, capsid and nucleoprotein core of an African swine fever virus (ASFV) particle. -- 5

Fig. 1.3 Map of ASF infected countries in Africa (1921-2011) based on reports -- 10

Fig. 1.4 Graphical description of ASF infected locations in Africa, epidemiological roles of wild/domestic pigs and the Ornithodoros ticks, and relationship with recent outbreaks in other parts of the world -- 11

Fig. 2.1 Distribution of wild suids associated with the incidence of African swine fever in Africa --- 19

Fig. 2.2 Probable route of spread of ASF within Nigeria 1997-1998 and 1999-dates based on available data --- 21

Fig. 2.3 Location of confirmed outbreaks and serological evidences of ASF in Nigeria (1997-2009) --- 22

Fig. 3.1 Political Map of Africa -- 36

Fig. 3.2 Map of Nigeria showing the different geopolitical zones and states -- 37

Fig. 3.3 Comparative ASFV antibody and genome detection in samples collected from selected states in Nigeria between October 2006 and April 2009. -- 45

Fig. 3.4 Regional comparison of ASFV antibody and genome prevalence between October 2006 and April 2009, involving 17 states and over 35 locations in Nigeria -- 46

Fig. 4.1 Model of intervention with biosecurity and no intervention in a 122-sow farrow-to-finish pig farm. --- 60

Fig. 5.1a Spatio-temporal representation of reported and confirmed outbreaks of ASF outbreaks in Nigerian states, 1997-2003 ------------------- 92

Page

Fig. 5.1b Spatio-temporal representation of reported and confirmed outbreaks of ASF outbreaks in Nigerian states, 2004-2009 -------------------- 93

Fig. 6.1 Expression of fractions of Acetone extracts of *A. korupensis* using three different expression methods. -------------------------------------- 125

Fig. 6.2 Gel image showing PCR results and the effect of A. korupensis on ASF virus. --- 127

Fig. 7.1 Mid-point rooted neighbour-joining tree depicting the *Thymidine kinase* gene relationship of ASF viruses -- 148

Fig. 7.2 Unrooted neighbour-joining tree depicting the *Thymidine kinase* amino acid sequence relationship of ASF viruses --------------------- 149

LIST OF TABLES

Page

Table 2.1 The situation of the pig industry and ASF in countries within West Africa sub-region (June 2008) -- 20

Table 3.1 ASFV serological and virological resultsof the major pig trading establishments in Nigeria located within 17 states and over 35 localities --- 38

Table 4.1 Farm targets for a 122-sow farrow-to-finish unit -------------------------------- 63

Table 4.2 Six-monthly depreciation rates (in percentage) in the value of pigs based on expert survey and prevailing prices ------------------------------ 66

Table 4.3 Mid-year values of stock and costs of other inputs over a three-year farm operation period --- 67

Table 4.4 Mid-year values of stock and prices of other outputs over a three-year farm operation period --- 68

Table 4.5 Profit margins per annum over the three-year operation period --------------- 69

Table 4.6 Potential losses associated with ASF outbreaks --------------------------------- 69

Table 4.7 List and costs of items needed for biosecurity ----------------------------------- 69

Table 4.8 Sensitivity analyses of the implementation of biosecurity against ASF and price changes in a 122-sow farrow-to-finish piggery ---------------- 72

Table 5.1 Population characteristics of the case and control farms participating in the study of ASF in Nigeria, 2008-2011 ---------------------- 94

Table 5.2 Univariable conditional logistic regression analysis of risk factors associated with presence of ASF outbreaks on pig farms, Nigeria, 2008-2011 -- 95

Table 5.3 Multivariable conditional logistic regression analysis of risk factors associated with ASF virus infection in a matched case-control study of pig farms, Nigeria, 2008-2011 ---------------------------- 96

Table 5.4 Univariable conditional logistic regression analysis of the association between self-reported biosecurity practices and the presence of ASF outbreaks on pig farms, Nigeria, 2008-2011 ------------ 97

Page

Table 5.5 Multivariable conditional logistic regression analysis of self-reported biosecurity practices against ASF outbreaks on pig farms, Nigeria, 2008-2011 -- 98

Table 6.1 Set up of the test system for the antiviral assay -------------------------------- 121

Table 6.2 Secondary metabolites found in the different stem barks, leaves and roots of the *A. korupensis* --- 123

Table 6.3 Compounds expressed from the stem barks, leaves and roots of *Ancistrocladus korupensis* --- 124

Table 6.4 Observation of activities of *Ancistrocladus korupensis* extracts and fractions on haemadsorbing African swine fever virus *in-vivo* 5-days post infection --- 126

Table 7.1 Summary of the viruses characterized in this study --------------------------- 145

LIST OF APPENDICES

Page

1. Appendix A: OIE Multi-annual ASF disease incidence (New Outbreaks, 1996-2004). --184
2. Appendix B: Global picture/Disease Timelines for African swine fever, 2005-2011 (OIE). --186
3. Appendix C: Questionnaire Survey on Impacts and Epidemiology of African swine fever in Nigeria ---191
4. Appendix D (1-3): Results of Gas Chromatography-Mass Spectrometric analysis of *Ancistrocladus korupensis* --------------------------------------195
5. Appendix E: Translated amino acids of *Thymidine kinase* genes of ASF viruses used in the study ---196
6. Appendix F: Translated amino acids of *Thymidine kinase* genes of ASF viruses used in the study ---200

LIST OF ABBREVIATIONS

%	: Percentage
^oC	: Degree centigrade
Ace	: Acetone
AIC	: Akaike information criterion
ASF	: African swine fever
ASFV	: African swine fever virus
BCA	: benefit-cost analysis
BCR	: benefit cost ratio
BEA	: Benzene-Ethanol-Ammonia
BIC	: Bayesian information criterion
CEF	: Chloroform-Ethyl acetate-Formic acid
Chloro	: Chloroform
$CI_{95\%}$	: 95% Confidence Interval
CIA	: Central Intelligence Agency
CISA-INIA	: Centro de Investigación en Sanidad Animal- Instituto Nacional de Investigación y Tecnologia Agraria y Alimentaria.
CLUSTAL W	: Multiple sequence alignment programme for DNA or protein
CPE	: cytopathic effect
CVR	: central variable region
DCM	: DiChloroMethane
DMBA-MBTH	: 3-Dimethylaminobenzoic acid+3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate
DMSO	: Dimethyl sulfoxide
DNA	: Deoxy ribonucleic acid
dNTP	: Deoxynucleotide
EDTA	: Ethylene di-amine tetra acetic acid
ECTAD	: The Emergency Centre for Trans-boundary Animal Diseases
ELISA	: Enzyme-linked immunosorbent assay
EMW	: Ethyl acetate-methanol-water
EMBL	: European Molecular Biology Laboratory
EMPRES	: Emergency Prevention System for Transboundary Animals and Plant Pest Diseases
ESAC-WA	: Europe, South America, the Caribbean and West Africa

ESRI	: Environmental Systems Research Institute
EU	: European Union
FAO	: Food and Agricultural Organization of the United Nations
FAOSTAT	: Food and Agricultural Organization Statistical database
FMA&RD	: Federal Ministry of Agriculture and Rural Development
GC-MS	: gas chromatography-mass spectrometry
HAD	: Haemadsorption
Hex	: Hexane
HRP	: Horseradish Peroxidase
i-ELISA	: Indirect enzyme-linked immunosorbent assay
IB	: immunoblotting assay
ICTVdB	: International Committee on Taxonomy of Viruses data Base
IU	: International unit
MeOH	: Methanol
MEGA	: Molecular evolutionary genetic analysis
NCBI	: The National Centre for Biotechnology Information
NS1	: Non-structural protein 1
NS2	: Non-structural protein 2
NVRI	: National Veterinary Research Institute
OD	: Optical density
OIE	: World Organisation for Animal Health
OR	: Odd Ratio
ORF	: Open reading frame
PBMC	: Primary bone marrow culture
PCR	: Polymerase chain reaction
PLC	: primary leukocyte cultures
pMol	: pico Mol
PSGA	: Penicillin, streptomycin, gentamycin, amphotericin B combination
RBC	: Red Blood Cells
RFLP	: Restriction fragment length polymorphism
q-PCR	: Quantitative polymerase chain reaction
SW Nigeria	: South-West Nigeria
TADP	: Transboundary Animal Disease Programme of the ARC-Onderstepoort Veterinary Institute, South Africa

TAE	: Tris-Acetate-EDTA
TLC	: thin layer chromatography
U	: Unit
UNDP	: United Nations Development Programme
USD	: United States Dollars
USAID	: United States Agency for International Development
USDA	: United States Department of Agriculture
VI	: Virus isolation
V	: Volts
VRD	: Viral Research Department

1.0 GENERAL INTRODUCTION

1.1 African swine fever epidemiology

African swine fever (ASF) is a highly contagious transboundary viral infection of pigs that has serious socio-economic implications for people's livelihoods, international trade and food security. It is transmitted through direct contact amongst infected pigs, ingestion of contaminated swill and feedstuffs, infected formites and through the Argasid ticks of the genus *Ornithodoros* (Penrith, Thomson and Bastos, 2004). The domestic pigs (intensively managed or free-ranged) are highly susceptible to ASF and morbidity and mortality can reach up to 100% (Penrith and Nyakahuma, 2000; Sanchez-Viscaino, Mur and Martinez-Lopez, 2012).

Similarly feral pigs (escaped domestic species) and European wild boars (*Sus scrofa*) are equally susceptible to ASF although in a lesser degree but with similar epidemio-pathological patterns compared to the domestic pigs (Sanchez-Viscaino, 2006), and this makes it very difficult to eliminate the infection once ASF becomes endemic in these populations. ASF may infect and cause inapparent infection in a range of wild suids including the common warthog (*Phacochoerus africanus*), the bush pig (*Potamochoerus larvatus*), the giant forest hog (*Hylochoerus meinertzhageni*) and the Red River Hog (*Potamochoerus porcus*) (see figure 1. 1a-f). Sub-clinical infection together with the presence of suitable tick vectors plays a major epidemiological role in the maintenance of the virus in warthog populations in particular and is responsible for many of the intermittent infections observed in eastern and southern African countries (Jori and Bastos, 2009).

ASF has no bias with regard to age and gender. Oro-nasal secretions post-contact with infected pigs is the main route of infection in susceptible pigs. Feeding on contaminated pork products including swill and garbage waste can also contaminate susceptible animals (Owolodun *et al.*, 2010). Competent vectors of the *Ornithodoros* genus are the main reservoirs of virus persistence and transmission. However, the maintenance of ASFV in the domestic pigs in the absence of *Ornithodoros* ticks is dependent on the existence of sufficiently large, continuous populations of pigs at a high density and with a constant availability of naïve hosts for new infections and further spread (Penrith and Vosloo, 2009). Aerosol transmission has been shown to occur only over very short distances (FAO, 2009).

The incubation period varies from five to 15 days. The virus strains differ in virulence (high, mild or low virulence), and based on clinical signs associated with virulence, the disease can occur in acute, subacute and chronic forms. Clinical disease in an outbreak situation is usually of the peracute or acute forms where the virus may be shed copiously, and morbidity and mortality rates within an affected holding may approach 100%. However, following milder forms of infection or sometimes an acute infection, viraemia may persist for several weeks or months in clinically recovered and apparently healthy pigs. Although serum of recovered pigs has been shown to contain relatively long-lived non-protective antibodies to heterologous ASFV, these antibodies may offer some degree of protection to homologous strains of the virus (Gomez-Puertas *et al*., 1996; Neilan *et al*., 2004; Penrith, Thomson and Bastos, 2004; Sanchez-Viscaino, Mur and Martinez-Lopez, 2012). Serologically positive sows will transmit antibodies to piglets through the colostrum (FAO, 2009). Some studies have, however, indicated that passively transferred antibodies and active immunity can protect against lethal challenge of ASF (Onisk *et al*., 1994; King *et al*., 2011). In sub-acute and chronically infected pigs, virus replication continues even in the presence of antibodies, hence the risk of infecting naïve pig populations. Previously sick and recovered animals may present a continuous risk especially in large herds not only because the virus may be shed and has been isolated from tissues for up to six months post-infection, but because of the large population of susceptible pigs and constant introduction of fresh supplies of susceptible animals into the herd (Valadao, 1969; Penrith *et al*., 2004; Penrith *et al*., 2007).
Clinical signs may include high fever (>40°C) usually with hyperaemia of the skin, depression and loss of appetite. Sows may abort at all stages of pregnancy and this may be a source of infection to other pigs (Penrith *et al*., 2004; FAO, 2009). However, clinical examination is not a confirmatory diagnosis for ASF. Pathological signs may include extensive haemorrhages in the lymph nodes, spleen and kidneys and these may serve as additional confirmation of the presence of ASF. However, laboratory evaluation remains the only means of confirmation for ASF (Penrith, Thomson and Bastos, 2004; FAO, 2009).

(a) Warthog (*Phacochoerus africanus*)

(b) Feral pig (*Sus scrofa*)

(c) Red River Hog (*Potamochoerus larvatus*)

(d) Giant Forest Hog (*Hylochoerus meinertzhageni*)

(e) Bushpig (*Potamochoerus porcus*)

(f) European wild boar (*Sus scrofa*)

Figure 1. 1a-f: Representative pictures of wild and domestic suids involved in the epidemiology of ASF in Africa

1a, c, d and **e** have been shown to be susceptible to infection and transmit the virus in the sylvatic cycle but without the associated fatalities due to the disease. **1b** and **f** can be infected and display significant clinical signs and death similar to what is observed in domestic pigs.

Courtesy, ASF: History, a presentation of the Centro de Investigacion en Sanidad Animal-Instituto Nacional de Investigacion y Technologia Agraria y Alimentaria (CISA-INIA), Valdeolmos, Spain at the National Veterinary Research Institute, Vom, Nigeria 12th-22nd July, 2008.

1.2 African swine fever virus characteristics

The ASF virus (ASFV) is a DNA arbovirus belonging to the genus Asfivirus and the family *Asfarviridae* (Dixon *et al.,* 2005). The ASFV genome consists of a linear double-stranded DNA molecule of 170 to 193 kilobase pairs with terminal inverted repetitions and hairpin loops (de Villiers *et al*., 2010; Chapman *et al*., 2011). A high degree of variability in genome size and restriction fragment patterns has been observed when different ASFV isolates are compared. Restriction enzyme site mapping (Wesley and Tuthill 1984) and sequence analysis of virus genomes (Yañez *et al*., 1995; Chapman *et al*., 2008; de Villiers *et al*., 2010) have established that the central region of the ASFV genome is relatively conserved but large length variations occur at the termini, particularly within the 40 kbp of the left end of the genome, but also within 15 kbp from the right end of the genome. Many of the length variations are associated with the losses or gains of copies within the multigene families. In addition, smaller length variations are associated with the number of tandem repeats located at loci both within coding regions and in the intergenic regions between genes (Aguero *et al*., 1990; Almazan *et al*., 1995; Sumption *et al*., 1990; Dixon *et al*., 1990; Rodriguez *et al*., 1994; Irusta *et al*., 1996).

Morphologically, the ASF virion consists of an envelope, a capsid, a core, and a nucleoprotein complex. The virions have an extracellular phase and can occur in two phenotypes (Dixon *et al*., 2005; ICTVdB, 2006). The virus capsid is enveloped and the virions are spherical and measure approximately 200-300 nm in diameter. Intracellular virions are not enveloped and the capsid/nucleocapsid is isometric with icosahedral symmetry and a diameter of 80 nm. Capsids appear hexagonal in outline. The capsid surface structure does not reveal a regular pattern with distinctive features and consists of 1892-2172 capsomers (with a diameter of 13 nm each, they are hexagonal prisms with a central hole with an intercapsomeric distance of 7.4-8.1 nm) (See Figure 1. 2; Dixon *et al*., 2005, ICTVdB, 2012).

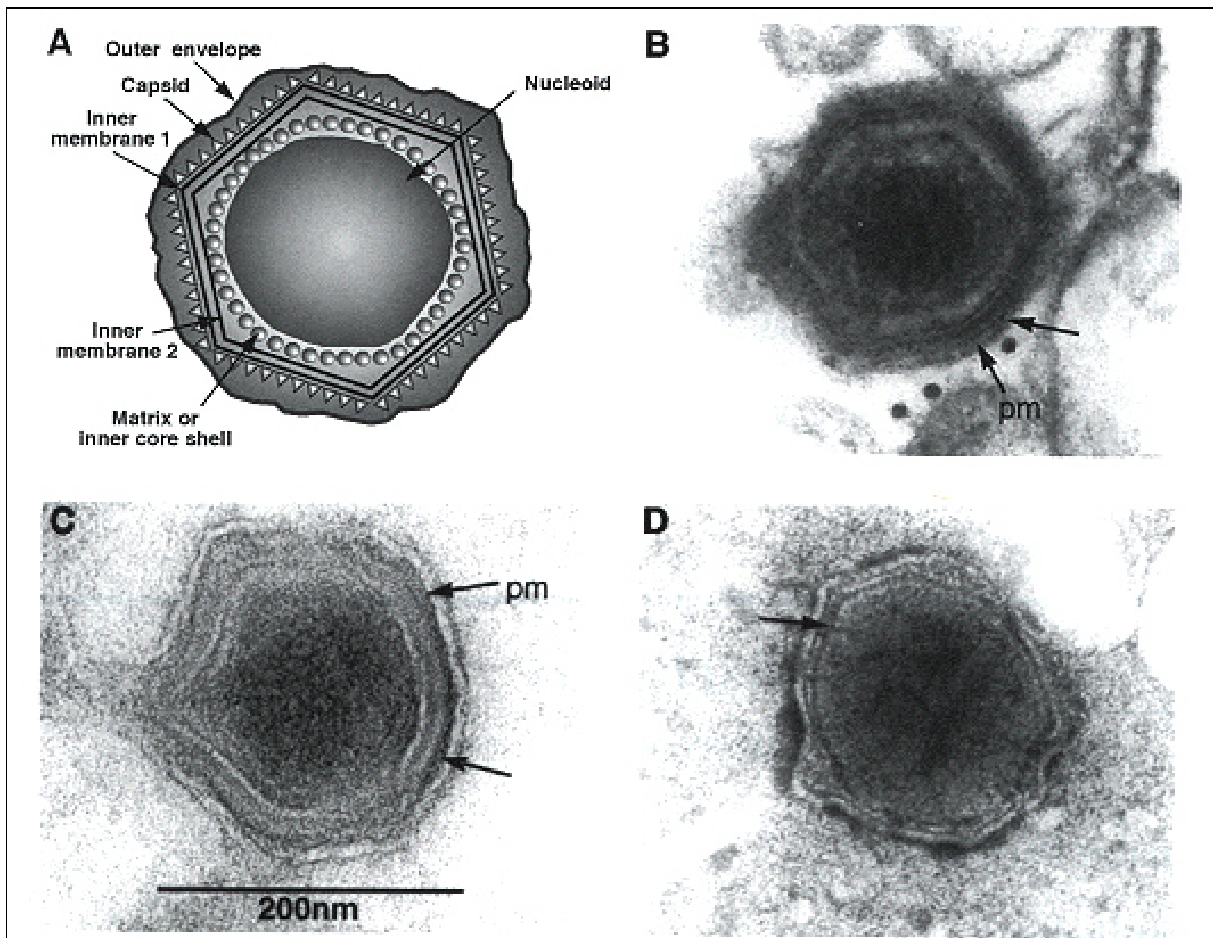


Figure 1. 2: Electon micrographs and schematic representation of the lipid membranes, capsid and nucleoprotein core of an African swine fever virus (ASFV) particle. (A) ASF virion contains viral-associated proteins (enzymes). (B) Thin section, (C) cryo-section and (D) a negative contrast electron micrograph of ASFV particles. The arrows indicate the membrane components of the virus; pm = plasma membrane. The bar length in (C) corresponds to 200 nm.
(EMs provided by Dr Sharon Brookes, IAH Pirbright.)
Copyright: Academic Press (2000) and International Committee on Taxonomy of Viruses (2002, accessed in March, 2012).

The lack of discernible ASF serotypes has necessitated that field strains be grouped genetically into discrete genotypes (Wesley and Tuthill, 1984; Blasco *et al*. 1989; Bastos *et al*., 2003; Lubisi *et al*. 2005; Boshoff *et al*., 2007). In earlier studies, the method of choice was restriction fragment length polymorphism (RFLP), which has been largely replaced by rapid PCR-based methods, such as C-terminus end of the *p72* gene, *a* genotyping which recovers the same major groupings (22 genotypes) as RFLP analysis (Bastos *et al*., 2003; Lubisi *et al*., 2007). This *p72* gene sequencing approach has revealed that ASF viruses causing outbreaks in Nigeria between 1998

and 2000 belong to *p72* genotype I (Odemuyiwa et al., 2000; Bastos *et al.* 2003), or more broadly classified as the Europe, South America, the Caribbean and West Africa (ESAC-WA) genotype which derives its name from the four ASF-free regions that it has made incursions into *viz.*, Europe, South America, the Caribbean and West Africa. The partial *p72* gene region that is advocated for virus genotyping (Bastos *et al.*, 2003), is characterised by extreme intra-genotypic homogeneity for pig-associated genotypes such as the ESAC-WA or genotype I, rendering this gene region of little use for tracing the origin and course of outbreaks in certain instances (Bastos *et al.*, 2003, Phologane *et al.*, 2005; Gallardo et al., 2009).

Intra- genotypic size and sequence variability in the central variable region (CVR) of the 9RL ORF (also termed pB602L) has, however, been demonstrated for genotype I viruses (Irusta *et al.*, 1996; Phologane *et al.*, 2005; Nix *et al.*, 2006, Owolodun *et al.*, 2010). Whilst alternative genes with greater intra-genotypic resolution capabilities than *p72* have been identified (Nix *et al.*, 2006; Gallardo *et al.*, 2009), these alternative genome targets are of limited use for geographically and temporally-constrained viruses such as those causing outbreaks in Kenya from 2006 to 2007 (Gallardo *et al.*, 2009; Owolodun *et al.* 2010). In a comparative study of three genome regions of genotype IX viruses recovered from outbreaks in Kenya in 2006 and 2007, only the CVR recovered more than one virus variant (Gallardo *et al.*, 2009). CVR therefore remains the genome target of choice when attempting to determine the origin and map the spread of closely related viruses (Owolodun *et al.*, 2010).

The ASFV, if present in a suitable protein environment is stable over a wide range of temperatures and *p*H; however, when it is not protected, the virus is rapidly inactivated by sunlight and desiccation. Due to this known tolerance to a wide range of *p*H (1.9 – 13.4), only certain disinfectants are known to be effective against the virus (Plowright and Parker, 1967). The ASFV is also resistant to the decrease of *p*H which accompanies the meat maturing process and it is not inactivated by freezing and thawing. The virus is similarly relatively stable in excretions of infected pigs, in pig carcasses, and in some pig meat products and fresh pig meat. Putrefaction does not necessarily inactivate the virus and it may remain infective in faeces for at least 11 days and in bone marrow for months (FAO, 2009). The ability of the ASF virus to remain infective in edible products such as chilled meat (at least 15 weeks and probably for as long as 300days) and for up to a year in cured hams and sausages that have not been cooked or smoked at a high temperature, has important implications for

the spread of ASF (McKercher *et al*., 1978; FAO, 2009). Undercooked, dried, smoked and salted pork and blood or carcasses and carcass meal derived from pigs in an outbreak or endemic setting must be regarded as potentially infective if fed to pigs and/or discarded in communal waste sites where pigs may feed. However, cooked or canned hams are safe, as long as they have been heated through at 70°C for more than 15 minutes (USDA-ERS, 2009).

1.3 Global spread of African swine fever

Historically, ASF was first described in Kenya by Montgomery (Montgomery, 1921) and in South Africa in 1928 (De Kock *et al*., 1940). The first isolate from the West African country of Angola was made in 1932 (Gago da Camara, 1932), followed by Senegal in 1959. Infection in the Gambia, Guinea Bissau and probably Nigeria date back to 1973, following which ASF disappeared from the region until the infection reappeared in Cameroun in 1982 and again in 1985 (Wesley and Tuthill, 1984; Ekue and Tanya, 1985; Bastos *et al*, 2003; Nix et al, 2006) as well as in Chad in 1983, 1984 and 1985 (Plowright, Thomson and Neser, 1994). Greater expansion of ASF commenced in West Africa in 1996 when Côte d'Ivoire became infected, followed by Benin, Nigeria, and Togo in 1997, Ghana in 1999 and 2002 and Burkina Faso in 2003. With the exception of Côte d'Ivoire, the disease has not been eradicated and numerous outbreaks have occurred since the introductions (See Figures 1. 3a-d & 1. 4; El Hicheri, 1998; El Hicheri *et al*., 1998; Penrith, 1998, Penrith and Vosloo, 2009).

It should be understood that at the time of the outbreaks, the Nigerian capacity (and those of other West and Central African countries) to promptly respond to and control such potential ASF outbreaks was untested , veterinary services were weak and the reporting systems deficient. Consequently, ASF spread rapidly along pig trading routes in Nigeria and the first wave of high mortalities was recorded between September, 1997 and October, 1998 (El Hicheri, 1998). An estimated 125,000 pigs from nine States were said to have been lost in this initial wave. Since this initial epizootic, subsequent waves (especially those occurring in 1999 and 2001) and sporadic outbreaks have persisted in Nigeria and this has had devastating impacts on both subsistence and commercial pig activities.

To date, despite the widespread devastation caused by the epizootics of ASF in Nigeria, and its attendant socio-economic effects, little progress has been made in the control and eradication of the disease in this West African country.

Although ASF has been widely documented in Nigeria, research, diagnostic efforts and reports have remained localized or at best regionalized (Odemuyiwa *et al.*, 2000; Otesile *et al.*, 2005; Babalobi *et al.*, 2007, Luther *et al.*, 2007b; Owolodun *et al.*, 2007) with the most spatially representative effort being a recent report by Owolodun *et al.* (2010). Whereas a previous report claimed that ASF virus may not be present in Nigerian warthogs and other wild suids, (Taylor *et al.*, 1977), recent evidence of the virus in warthogs and red river hogs, possibly due to spillover infections from pigs (Luther *et al.*, 2007a; Luther *et al.*, 2007b), indicates that wild suids can no longer be discounted as potential role-players in the epidemiology of the disease in West Africa.

To date, ASF remains endemic in Nigeria but the situation is unknown in most other West and Central African countries. Ghana and Côte d'Ivoire have controlled the infection within their territories. Recently outbreaks were reported in Cape Verde, Togo, Ghana, Southern Chad, Kenya, Tanzania, Uganda, South Africa and also Cameroun (World Animal Health Information System, 2012).

In other parts of Africa, ASF has been widely demonstrated in East and southern African territories including Angola, Botswana, Burundi, Kenya, Malawi, Mozambique, Namibia, Rwanda, South Africa, Tanzania, Uganda, Zambia and Zimbabwe (Bastos *et al.* 2003; Bastos *et al.*, 2004; Lubisi *et al.* 2005; Boshoff *et al.*, 2007; Lubisi *et al.*, 2007; OIE, 2010) and has in recent decades extended its epizootiological range to include the Indian Ocean islands of Madagascar and Mauritius (Gonzague *et al.* 2001; Lubisi *et al.* 2009). Though there are no reports of the presence ASF in North African countries, the disease has been recorded in Chad (Figures 1. 3a-d; Plowright, Thomson and Neser, 1994).

Outside the African continent, ASF was first reported in 1957 in Portugal, apparently introduced from Angola (Wilkinson 1989) and re-emerged in 1960 (Manso-Ribeiro and Azevedo, 1961). This second introduction was not controlled and the disease subsequently spread to other European countries including Spain, Belgium, France, Italy, Malta and the Netherlands (Polo and Sanchez, 1961; McDaniel, 1986; Biront *et al.*, 1987). In the late 1970s and early 1980s, the disease spread to the Caribbean and South American nations of Cuba, the Dominican Republic, Haiti, and Brazil (Bram *et al.*, 2002, McDaniel, 1986, Rendleman and Spinelli, 1999). These incursions

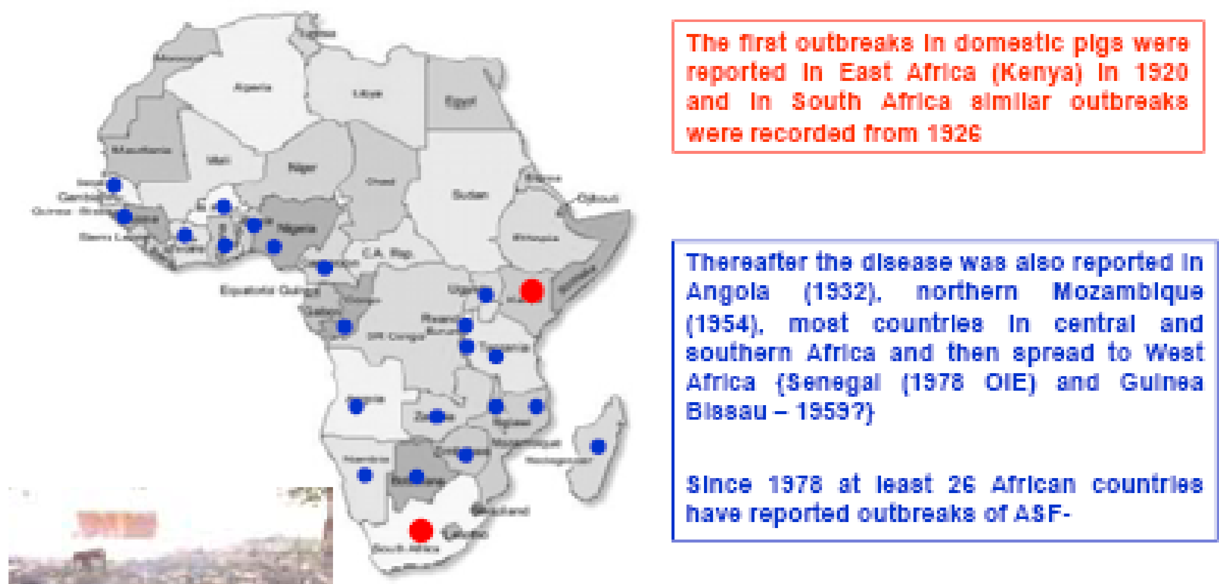
demonstrated the devastating effect of ASF on pig production and potential for rapid transboundary spread. The disease remained endemic in Europe for almost 42 years, and following stringent and expensive control measures, ASF was eradicated outside the African continent, with the exception of the Italian island of Sardinia where since 1982 it remains enzootic (Dixon *et al*., 2005, OIE disease information sheet).

On May 2007, the first clinical cases of ASF were seen in the area surrounding port Poti on the eastern shore of the Black Sea, in Georgia, although the disease was not reported to the OIE until 5 June 2007 (Rowlands *et al*., 2008). Afterwards, the disease spread eastwards and northwards following the main transportation routes. This was the first official report of ASF occurrence in the Caucasus region. Sequence analysis of the Georgia ASF virus isolate revealed a close relationship to virus strains from South-East Africa (Mozambique, Madagascar and Zambia) (See Figure 1. 3 and Appendix A; Rowlands *et al*., 2008). Since this introduction, ASF has spread to neighbouring countries of Armenia, Azerbaijan, Abkhazia and Nagorno- Karabakh. In November 2007, the disease was first reported in the southern regions of Russia federation where a previous infection had been eradicated in 1977, and it has slowly spread north and west in domestic pigs and to the east in wild boar. On October 2009 ASF jumped approximately 2,000 kilometres from southern Russia to St Petersburg in north-western Russia. (Rowlands *et al*., 2008; FAO, 2009) and was also confirmed in wild boars in Iran (Rahimi *et al*., 2010). It is presently causing high fatalities in pigs in Russia and the Caucasus (Callaway, 2012). The complexities arising from the domesticated and wild pig (European wild boar) interface and ongoing infections is of concern to Europe and poses a significant threat to Asia and China in particular, which has over 50% of the world's pig population (EFSA, 2009; EFSA, 2010; Callaway, 2012). Furthermore, a recent study highlighting this risk has confirmed that the European Union stands a chance of experiencing ASF outbreaks should the current situation continue unabated (Mur et al., 2012)

To date countries in North America, Asia and Oceania have never report an infection with the ASFV (Thomson, 1985, Rendleman and Spinelli, 1999). These countries, which all have well-developed pig industries and are currently free of the infection, have taken serious measures to prevent its entry (Miller *et al*., 1996).

Figure 1. 3: Map of ASF infected countries in Africa (1921-2011) based on reports

Countries highlighted in red have reported outbreaks or are endemic for ASF. Countries not highlighted have never reported infection with ASFV. However, it is possible that suspect cases may have occurred in some of these countries, especially in Guinea and Liberia.

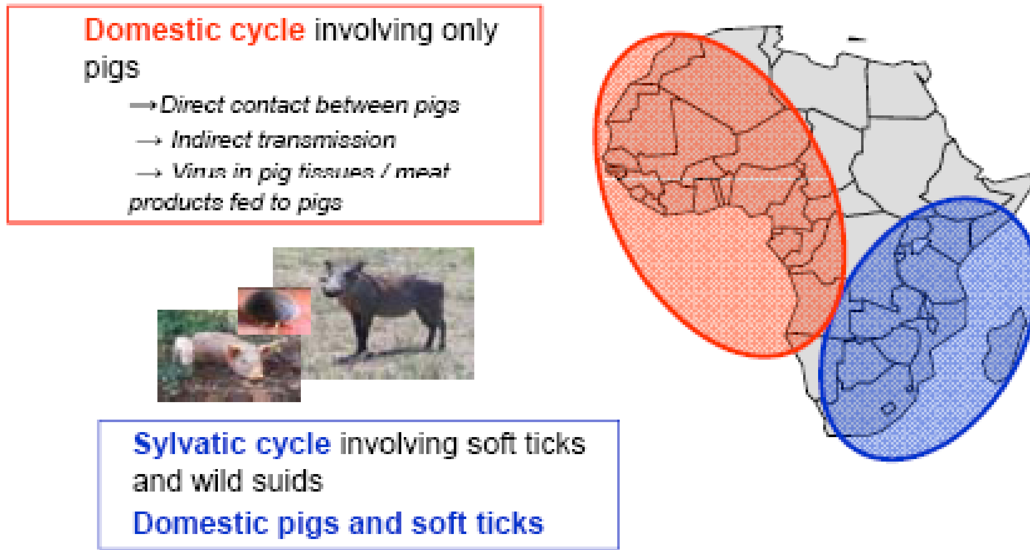


(a)

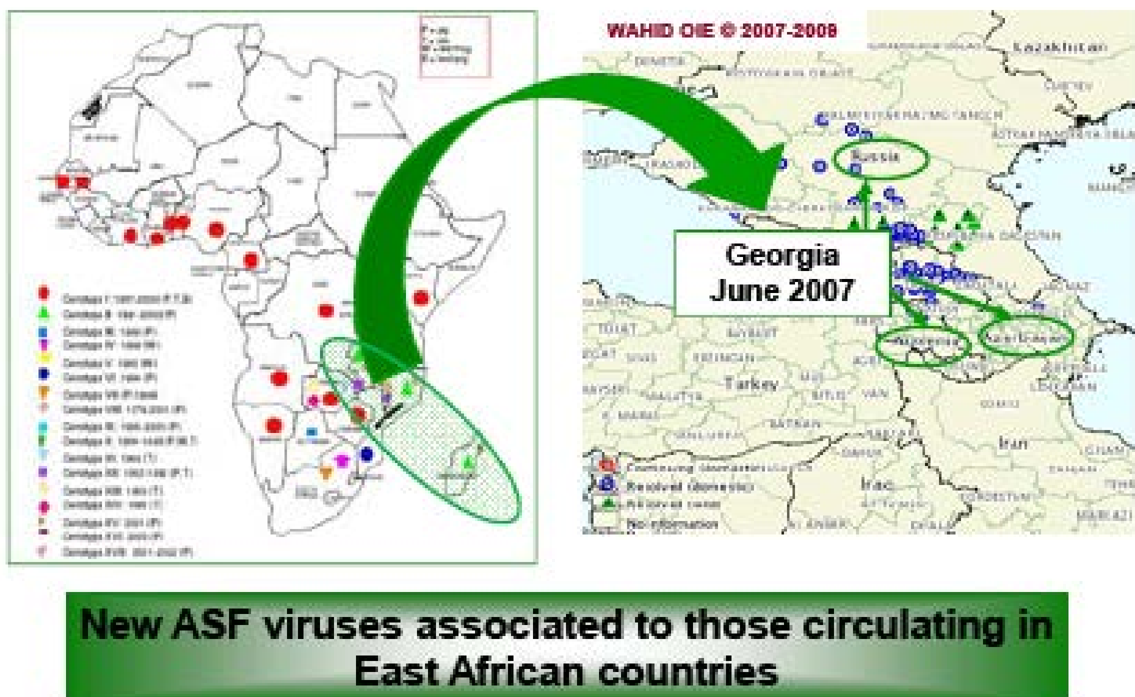
(b)

Figure 1. 4a & b: Graphical description of ASF infected locations in Africa, epidemiological roles of wild/domestic pigs and the *Ornithodoros* ticks, and relationship with past and recent outbreaks in other parts of the world

Courtesy: C Gallardo, A general view on ASFV molecular epidemiology and diagnostic research. Compultense University, Madrid, Spain. 12 July, 2010



(c)



(d)

Figure 1. 4c & d: Graphical description of ASF infected locations in Africa, epidemiological roles of wild/domestic pigs and the *Ornithodoros* ticks, and relationship with past and recent outbreaks in other parts of the world
Courtesy: C Gallardo, A general view on ASFV molecular epidemiology and diagnostic research. Compultense University, Madrid, Spain. 12 July, 2010

1.4 Sources of Infections and Management strategies

Empirical evidence and spatial distributions of the epizootic of ASF in West Africa pointed to the movement of infected pigs (and their products) including those of contaminated materials (Olugasa and Ijagbone, 2007); and the clear absence of audited biosecurity programmes, as the most important factors contributing to the continuous spread and dissemination of ASF in parts of the sub-region. The farmers often engaged in the slaughter of pigs within the farm premises, sold the sick and unthrifty pigs at markets, allowed farm-gate buyers (who visit numerous pig farms) free access to their facilities and patronized unorganized markets. This situation is supported by weak veterinary infrastructures and lack of effective capacity to rapidly diagnose and contain the spread of the virus (El Hicheri *et al*., 1998).

Although the warthogs (*Phacocoerus africanus*) population are sparse throughout the West African savannah, with limited opportunity for contact with free-ranging domestic though possibly with feral pigs, there is presently no tangible evidence for their role in the epidemiology of ASF in the region, aside from probable spillover infections (Penrith, 1998; Luther *et al*., 2007a; Luther *et al*., 2007b; Jori and Bastos, 2009). Similarly, eyeless ticks of the *Ornithodoros moubata* complex have never been recorded from West Africa (Leeson, 1958; Ekue and Wilkinson, 1990). The family relative, *O. erraticus*, which acted as a vector for ASF in Spain and Portugal, has been reported from Senegal and other sahelian countries including Chad, but is not known to be associated with warthogs. One naturally infected *O. sonrai* tick was, however, identified in Senegal confirming a possible role for this tick species in virus maintenance (Vial *et al*., 2007).

Bush pigs (*Potamochoerus porcus*) occur in the forested areas of West Africa, where traditional pig production is concentrated, as well as in the areas of known endemicity in central Africa. They are known to be susceptible to infection with ASF virus but resistant to the disease (Plowright, Thomson and Neser, 1994). However, their role in the epidemiology of ASF in West Africa, if any, is unknown.

During past ASF outbreaks in Nigeria in 1997, farmers sometimes resorted to managing the fatal consequences of ASF using unorthodox methods and ethno-veterinary preparations. These approaches which have widely varied claims of

effectiveness are still in use in the country. The lack of subsidies for animal agriculture in Africa has necessitated that alternative therapy must be sought by several resource-poor small-scale pig farmers to save their stock or the remaining surviving stock in an outbreak situation. One approach with potential benefits of reduced morbidity and mortality, and sometimes complete freedom from illness post-treatment was the administration of preparations from plant sources. *Ancistrocladus korupensis* recently described by Thomas and Gereau (1993) is a Liana plant. The plant is found mainly in the tropical swamp of Korup National Park in Cameroun and the adjoining Cross River National Park in Nigeria. Its population structures and biological/phytotherapeutic potentials (including known antiviral activities) have been studied extensively (Boyd et al., 1994; McMahon et al., 1995; Foster and Sork, 1997) but no assessment of this plant material has been carried out against the ASF virus of pig or any other animal virus. Empirical support for the claims that ASF can be successfully treated with extracts from this plant is presently lacking.

1.5 Role of Pigs and Economic Losses Associated with ASF

Pigs represent an important economic source of income globally. The FAO data on global meat consumption for 1999 revealed that 88 million tonnes of pig meat was consumed making it the number one meat by quantity consumed for that year (Moore, 2007). National and international data on pigs and pork consumption are in agreement with the FAO reports. Since 1995, the USA has become a major net exporter of pork, with an export market worth of over $1,000 million in 1995 alone (USDA-ERS, 2009, National Pork Producers Council, 2010). However, to date, China remains the most important role-player in the pig industry having approximately 50% of the global pig population. In Benin, a country in West Africa with a human population of approximately 7.9 million (CIA World Fact Book, July, 2006 estimate), an organization has been involved in a major pig production project (Songhai project) targeted at providing pork and other pig products for about 20% of the population. In Côte d’Ivoire, pork constitutes 15% of the total meat consumption and is a widely available source of cheap meat. The estimated pig population in 1996 was 400,000 for this country.

In Nigeria, the estimated pig population increased from nearly 2 million in 1984 to about 7 million in 1997 (El-Hicheri, 1998). This rapid increase was associated with the government agricultural initiative aimed at boosting agricultural production and alleviating poverty among the rural and urban poor. Benue, a state in north-central Nigeria with an estimated human population of 2,780,398 (1991 national census figure) alone has about 27,000 pig farmers (≈0.001% of the state population) and produces about 20% of the country's pigs annually. Major cities and rural areas in the southern parts of the country similarly have numerous pig farms and farmers' cooperatives. The poor and smallholder farmers who raise an average of 1-50 pigs own the majority of the Nigerian pig population. The continued rise in demand for pigs and pig products in the Lagos metropolis and its suburbs as well as in most other upcoming cities prior to the ASF outbreaks of 1997 boosted pig production activities.

Secondary to the purely economic benefits associated with pig production, pigs are used for socio-cultural reasons including bride price, marriage ceremonies, rituals and funeral rites. Furthermore, its by-products serve as a source of fertilizer and energy generation for farmstead and household needs (El-Hicheri, 1998).

The advent of ASF in 1997 and the consequences as reported above has continued to delimit the successes associated with the progress made in the industry. Many pig farmers were discouraged and abandoned pig production while the farmers who continue to produce pigs stand the perpetual risk of ASF outbreaks. To date, it is difficult to determine the actual pig population in Nigeria in view of this perpetual incursion and sporadic outbreaks of ASF on pig farms and the resulting fluctuating levels of commitment to the industry.

1.6 AIM AND OBJECTIVES

1.6.1 Aim

The aim of the research is to determine the current national prevalence of ASF in Nigeria, establish the key epidemiological concepts and risk factors that support the introduction and perpetuation of African swine fever at farm-level, use available molecular diagnostic tools to explore the virulence of viruses from West Africa and other parts of world, and to evaluate the ethnoveterinary potentials of a selected plant,

its extracts and its fractions in order to explore the the implications and feasibility of applying structured biosecurity at farm-level.

The study will assist with understanding the eco-epidemiology of ASF and should positively influence the management and approaches for ASF control in West Africa using a combination of epidemiological and sound socio-economical principles (Fournie, Rushton and Pfeiffer, 2012).

1.6.2 Objectives

1. To determine the national and location-based prevalence of ASF in the different agro-ecological zones of Nigeria.
2. To relate temporally and spatially, ASF isolates from the different regions of Nigeria and determine epidemiological links and risk factors.
3. To conduct an informed study on the cost of ASF to smallholder piggeries and to determine whether the cost of alternative approaches to disease control is justifiable for smallholder farmers.
4. To determine if field claims of effectiveness of ethnoveterinary medicine in the management of ASF holds any potential for treatment of the disease, using the *Ancistrocladus korupensis* plant as an *in vitro* experimental model.
5. To compare and contrast the thymidine kinase virulence gene of ASF field strains, across genotypes in relation to virus pathogenicity as a basis for ASFV classification.

Considering the importance of the disease and economic devastation it has caused the pig industry, the achievement of the above listed objectives will enable better policy planning, control and management options to sustain the pig sector of the livestock industry in Nigeria, in particular, and to West Africa in general.

2.0 RATIONALE AND APPROACH TO THE STUDY

2.1 ASF Epidemiology in West Africa

From 1996 to date, African swine fever has gained an epizootic dimension in West and Central Africa. It was first reported in Cote d'Ivoire in 1996 leading to the death of over 135,000 pigs (El Hicheri *et al.*, 1998). Prospective and retrospective evaluation of notifications, diagnostics and events associated with ASF outbreaks has revealed that the disease was first discovered in Africa in Kenya in 1921 (Montgomery, 1921); that it occurs in many eastern and southern African countries, including:f Angola, Botswana, Burundi, Kenya, Malawi, Mozambique, Namibia, Rwanda, South Africa, Tanzania, Uganda, Zambia and Zimbabwe (Edelsten and Chinombo, 1995; Bastos *et al*. 2003; Bastos *et al*., 2004; Penrith, Thomson and Bastos, 2004; Lubisi *et al*. 2005; Boshoff *et al*., 2007; Lubisi *et al*., 2007; OIE, 2010); it was present in Senegal in 1959 (Bastos et al., 2003), Cameroun in 1982 and 1985 (Wesley and Tuthill, 1984; Ekue and Tanya, 1985; Nix et al., 2006) and many other West Africa countries (Benin, Togo, Ghana, Gambia, Senegal, Cameroun, Chad and Cape Verde) between 1996 to date (Plowright, Thomson and Neser, 1994; El Hicheri, 1998; El Hicheri *et al*., 1998; Odemuyiwa *et al*., 2000; Penrith, Thomson and Bastos, 2004; Vial *et al*., 2007). Recent outbreaks have been reported from Madagascar and Mauritius (Gonzague *et al*. 2001; Lubisi *et al*. 2009), and more recently in Chad, Cameroun, Tanzania, Kenya, South Africa and many other locations within and outside Africa (See Appendix B., OIE, 2012).

It will be technically sound to consider the whole of the West African sub-region as one epidemiological block and approach the management of any animal disease situation sub-regionally in view of the following:

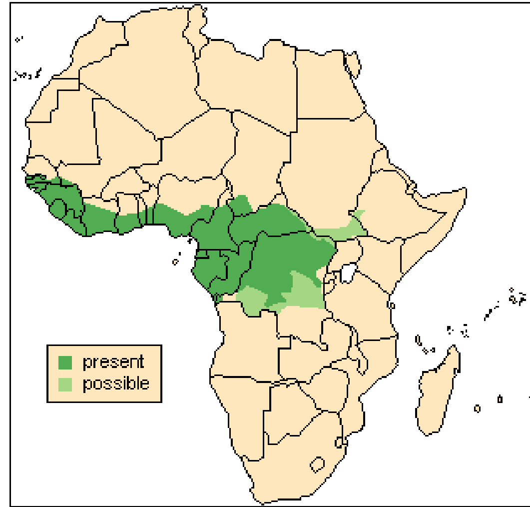
1. There are no strict border restrictions in the whole of the sub-region and animals, including feral pigs, cross freely within and between local and international borders.
2. The free trade zone policy operates in the sub-region and the livestock markets within the countries in the sub-region are freely accessible to traders and farm-gate buyers within the sub-region. In addition, these markets are typically located close to or along the porous borders and illegal trade occurs daily.

3. Quarantine stations, laboratory services and veterinary investigative capacities are weak or non-existent.
4. The livestock disease ecology of the countries within the sub-region is similar with respect to their shared tropical climate, absence of ticks of the *Ornithodoros moubata* complex, and unconfirmed/dwindling populations of wild pigs. Further, no direct role has been assigned to wild pigs in the epidemiology of ASF in West Africa.

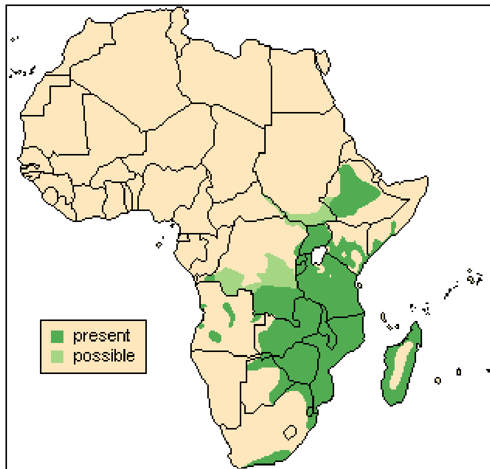
It will appear that the West African sub-region was poorly prepared for the outbreaks that started in 1996. Following the initial infections and subsequent outbreaks in Côte-d'Ivoire, the FAO sent notifications to neighbouring countries, however, the lack of an established and tested early-warning-system that can trigger immediate reactions and prompt control and management of rapidly spreading infectious diseases prevented the gains that should have been associated with such notifications (El Hicheri, 1998). Furthermore, there was a rapid growth of the pig populations across the sub-region in the face of absent zoosanitary measures (movement restrictions, road blocks, quarantine stations, closure of livestock markets, prompt stamping out and intensive epidemio-surveillances).

The distribution of wild pigs spans West and Central Africa (see Figure 2.1a-d) and largely coincides with the distribution of the disease. However, since there was no evidence to suggest that the classical sylvatic cycle which involves wild suids and ticks of the *Ornithodoros moubata* complex, played a role in the epidemiology of ASF in West Africa, these wild host were not believed to be responsible for the outbreaks recorded in the West and Central African region (Leeson, 1958; Ekue and Wilkinson, 1990). Recently, the soft tick species, *O. erraticus*, which acted as a vector for ASF in Spain and Portugal, was reported from Senegal and other sahelian countries including Chad, without any known association with warthogs; and *O. sonrai* was shown to be a possible role-player in the maintenance of the virus in parts of West Africa (Vial *et al.*, 2007). In light of this isolated report, and the growing body of literature substantiating a pig-exclusive cycle in the region, it will be scientifically sound to concentrate effort on domestic pigs in the understanding of epidemiology of ASF in West Africa.

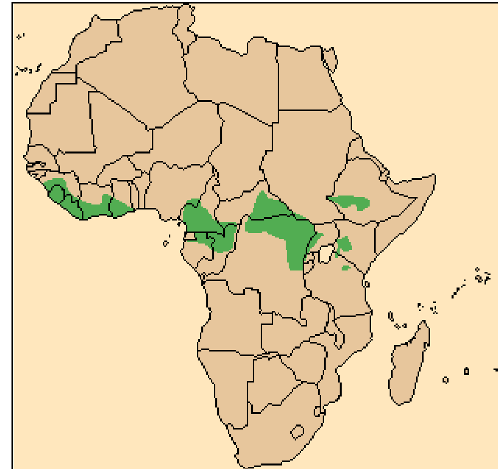
2.1a. Distribution of warthog (*Phacochoerus africanus)*



2.1b. Distribution of bushpig (*Potamochoerus porcus)*



2.1c. Distribution of red-river hog (*Potamochoerus larvatus)*



2.1d. Distribution of giant forest hog (*Hylochoerus meinertzhageni*)

Figure 2.1a-d. Distribution of wild suids associated with the incidence of African swine fever in Africa

The wild suids species have been confirmed to be present in the locations highlighted in green; areas marked in brown in 2.1a above have reported range or accidental records of warthogs. Other brown areas in 2.1b-d have not reported the presence of the wild suids.

Source: Vercammen et al., 1993; IUCN and Wikipedia.

In June 2008, an expert panel was set-up to review the West African sub-regional preparedness for rapidly containing and managing an ASF epizootic and the panel deemed the pig populations within the sub-region to be at a high risk of infection with ASF (see Table 1, LeFevre, 1998; El Hicheri, 1998).

Table 2. 1: The situation of the pig industry and ASF in countries within West Africa sub-region (June 2008)

Country	Status	Pig population prior to the 1996-1999 epizootics	Recorded fatalities + pig heads affected	Description of pig industry
Benin	Epizootic	>623,000	≈350,000 +42,000	Small
Togo	Epizootic	≈210,000	≈207,500 + 2,500	Small
Senegal	Enzootic	≈191,000	≈191,000	Small
Gambia	Enzootic	>65,000	≈65,000	Small
Guinea Bissau	Enzootic	≈25,000	≈25,000	Small
Nigeria	Enzootic	>7,000,000	125,000 -7,000,000	Large
Cameroun	Enzootic	>2,000,000	>600,000	Large
Ghana	Free with high risk of introduction	≈430,000	≈430,000	Small
Burkina Faso	Free with high risk of introduction	≈584,000	≈584,000	Small
Côte-d'Ivoire	Free with low risk of re-introduction	≈464,000	≈329,000	Small
Guinea	Free with low risk of re-introduction	≈45,000	≈45,000	Small
Liberia	Free with low risk of re-introduction	≈150,000	≈150,000	Small
Cape Verde		≈70,000	??	Small

Source: LeFevre, 1998; El Hicheri, 1998

These experts concluded that in the event of an outbreak, the capacities of most countries within West Africa to respond using a validated epidemiological system and good extension network were limited and reviews of such potentials has indicated that only Côte d'Ivoire has some capacity for such responses (El Hicheri et al., 1998). During the outbreaks in the West African sub-region, certain parameters / characteristics over and above those mentioned previously have been identified as potential risk factors including proximity of pig communities and villages to one another, avoidance of compulsory slaughter of affected herds, unorganized marketing networks and butchering of sick pigs, lack of compensation and extension services to support epidemio-surveillance, amongst others (El Hicheri, 1998). To date, none of the potential risk factors have been empirically examined, nor has the role of other professionals in the animal industry been investigated.

Specifically in Nigeria, the outbreaks first started in certain towns of the Ogun and Lagos states that border the Republic of Benin at the Queme Department (El Hicheri, 1998). The virus involved in this earlier outbreak was later characterized and linked to those from other outbreaks in West Africa (Odemuyiwa *et al.*, 2000). From these initial foci of infection, it rapidly spread causing the death of 125,000 pigs in nine states. The cause of this spread is possibly linked to the trade of infected pigs and

pork products since the affected states are linked by roads and trade and market in pigs and its products (see Figure 2.2 and 2.3, Fasina *et al*., 2009).

Legend		Initial entry point		Pig trade movement		Outbreak locations

Figure 2. 2. Probable route of spread of ASF within Nigeria 1997-1998 and 1999-dates based on available data.

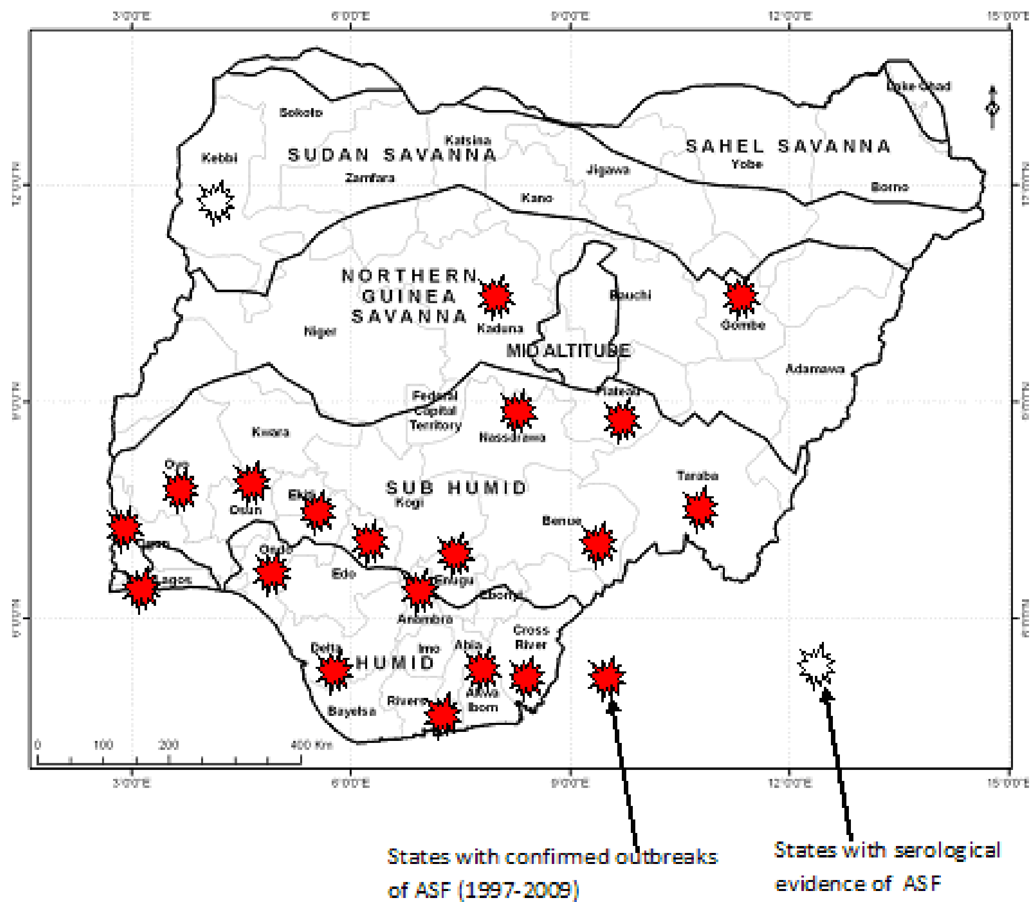


Fig 2. 3. Location of confirmed outbreaks and serological evidence of ASF in Nigeria (1997-2009)

Source: Fadiga, Jost and Ihedioha, 2011

Since the time of these initial outbreaks, several other studies have been carried out using serology, genetic analysis and virology to determine regional and locality-specific prevalence of ASF and its epidemiological and economic consequences. Serologically, ASF prevalence has been set at 7.5% and 12.8% for Plateau and Kaduna states respectively (Luther *et al*., 2002); 49.7% for Plateau state (Owolodun *et al*., 2005); 50% , 52.5%, 59.8%, 60.7% and 70% for Ondo, Oyo, Lagos, Ogun and Osun states respectively (Olugasa, 2007) and 55% for Kebbi state (Bala *et al*., 2009). Similarly, pooled tissue samples have indicated that ASF is prevalent in at least 14 states of Nigeria with an overall prevalence figure as high as 51% being suggested (Majiyagbe *et al*., 2004; Owolodun *et al*., 2007).

Though previous studies have indicated that all of the Nigerian isolates are grouped within the genotype I (ESAC-WA) using the *p*72 gene sequences and this has made discrimination of variants within the group difficult (Odemuyiwa *et al*., 2000; Gallardo *et al*., 2009), recent evidence suggests that six variants exist in Nigeria using

the central variable region (CVR) of ASF as a basis of genetic analysis (Nix *et al.*, 2006; Owolodun et al., 2010).

For the remaining parts of West Africa, peer-reviewed literature on the prevalence of ASF is limited. However, a recent report indicated that ASF prevalence in Senegal was 16.9% in 2006 and that prevalence ranged from 13.3%, 7.8% and 22.1% for the localities of Fatick, Kolda and Ziguinchor, respectively (Etter *et al.*, 2011).

2.2 Potential Risk factors for African swine fever

Risk factors and risk analysis studies for ASF have been carried out by many authors with important outcomes. The abundance of ticks of the genus *Ornithodoros* combined with the availability of wild suids has been known to complicate the epidemiology of African swine fever in regions where they co-occur (Haresnape, Wilkinson and Mellor, 1988; Anderson *et al.*, 1998; Roger *et al.*, 2001). As previously emphasised, the vectors as well as the wild suids have not been proven to date a role in the epidemiology of the disease in West Africa, to date.

Other studies postulate that herd size may be a risk factor for swine diseases including the ASF, however, confirmation for this should be correlated with the true population at risk, management-related factors, herd density, biological plausibility and herd distributions (Gardner, Willeberg and Mousing, 2002). In the case of West Africa, as is the case in most parts of Africa, the pig herds are small in size (LeFevre, 1998; Saka, Adesehinwa and Ajala, 2010; Nwanta *et al.*, 2011), hence, herd size may not truly reflect as a major risk factor for ASF in the sub-region.

Although, husbandry method has also been associated with seropositivity in pig herds (Madec and Rose, 2003) and Mannelli *et al.* (1997) proved that free-range pigs are five times more likely to be seropositive to ASF virus antibodies compared with pigs kept in intensive facilities, this factor has yet to be investigated in West Africa. In addition, the payment of compensation was suggested to have a positive impact on prompt disease reporting and a negative influence on subsequent disease spread following infection in Sardinia (Mannelli *et al.*, 1998). However, such compensation schemes are largely lacking in West Africa and may thus serve as a risk factor for the spread of the disease.

The legal and illegal sales of pig and pork products have been implicated in the transmission of ASF. Fresh pork, partially cooked and cured ham have been known to harbour the virus (Mebus *et al.*, 1993; Woolridge *et al.*, 2006). In Kenya, grazing

management and proximity to the wildlife parks are identified risk factors. These factors explained the role of wild suids in the epidemiology of ASF in East Africa but these may not be true for West Africa where wild pig populations are dwindling and presence of *Ornithodoros moubata* complex ticks have not yet established (Okoth *et al*., 2009). Within the European Union, the free-range pigs are at higher risks of infection with ASF virus and this risk is associated with swill feeding (EFSA, 2010).

In Denmark, inter-farm transportation and unrestricted movement associated with lack of barriers between the animal area and the loading bays have been identified as risk factors for contamination of herds (Boklund, Mortensen and Houe, 2005). In the course of the outbreaks in West Africa, poor enforcement of movement restrictions, sale of pig and pig products and lack of a ban on live pig markets encouraged the continued redistribution and rediffusion of potentially infected pigs and meat arising and increased the potential of contaminating “clean” farms (El Hicheri, 1998; El Hicheri *et al*., 1998; LeFevre, 1998). Similarly, the indiscriminate disposal of slaughtered pig viscera is a potential source of contamination to naïve herds and feral/free-range pigs can regularly contract infections from these infected viscera and disseminate the infection to other pigs. These are all activities that are likely to have played a role in the epidemiology of the disease in the sub-region.

Finally, considering the volume of illegal meat transportation within and outside the sub-region, ASF will continue to be a constant threat to other parts of the world (Woolridge *et al*., 2006; Chaber *et al*., 2010)

2.3 ASF and biosecurity in pig farms

“Biosecurity is the implementation of measures that reduce the risk of the introduction and spread of disease agents by the adoption of a set of attitudes and behaviours by people and managers to reduce risk in all activities involving domestic, captive/exotic and wild animals and their products” (FAO/OIE/World Bank, 2008). The necessity of biosecurity in pig operations especially for developing countries and those in transition has been emphasised by a joint Commission of the FAO and OIE (FAO/OIE, 2010). It remains the key to prevent the entry of infectious agents and contaminated/contagious materials into pig farms from outside sources (bioexclusion) and restrict pathogens within a farm where they are already present (biocontainment) using the three elements of segregation, cleaning and disinfection (Pritchard, Dennis

and Waddilove, 2005; Lambert and D'Allaire, 2009; Noremark, Frossling and Lewerin, 2010).

The complexities of biosecurity measures that are applicable to farms and whether the a farm-based or community-based approach to biosecurity should be applied will depend largely on the farm types (scavenging/free-range, backyard, small-scale, commercial, breeder or large outdoor), the geography, aggregations of farms and the socio-economic factors of the farmers (FAO/OIE, 2010). Control of most of the notifiable and rapidly spreading infectious diseases of animals will benefit from the application of farm-level and community-based biosecurity (Pritchard, Dennis and Waddilove, 2005; Lambert and D'Allaire, 2009). However to date, its implementation and adoption in pig farms across the world, and particularly in the West African sub-region, is rarely practised or evenly applied.

In a survey of 609 pig farms in Belgium, despite the industrialised nature of the pig farming systems, certain lapses in biosecurity (people not showering and the non-insistence of periods of quarantine) and different degrees of application were still observed (Ribbens *et al*., 2008). Similarly, in a survey of 172 piggeries in Spain, despite the fact that farmers were aware of the importance of biosecurity to reduce chances of infection in their herds, the application of on-farm biosecurity were at some variance with the knowledge of the subject (Casal *et al*., 2007). Vaillancourt and Carver (1998) had earlier outlined the difference between farmers' perceptions and the implementation of biosecurity measures in farms. Certain workers had emphasised the regular checks, review and validation of farm-based biosecurity to ensure its effectiveness (Nespeca, Vaillancourt and Morrow, 1997).

In Chile, though there was compliance with certain biosecurity issues in a survey amongst 50 large pig herds, breach of certain components of biosecurity procedures were also observed (Pinto and Urcelay, 2003). Similarly, in Denmark, thorough cleaning of pig farm vehicles transporting animals between farms as well as disinfection were identified as areas that needed improvement in the SPF and commercial farms (Boklund, Mortensen and Houe, 2005); there was no significant difference between the sow herds and the fattening herds although the SPF herds got higher biosecurity scores (Boklund et al., 2004, Boklund, Mortensen and Houe, 2005). The reports concluded by advocating compliance with quarantine procedures.

Within the African continent, Costard *et al*. (2009) recently reported on the assessment of biosecurity in 709 pig farms in Madagascar. Results indicated that

widespread poor biosecurity and geographical variations in its implementation exist. To date, there has been no single evaluation from the West African sub-region.

It should be known that the nationwide adoption of biosecurity and strict control of animal disease in any country is dependent on certain factors including:

- Globalization- faster movement, newer routes and associated trades in animal and animal products and how this affects the country.
- Conflicts and civil unrest- and its associated lack of enforcement of quarantine, difficulty in surveys, refugee movement sometimes with their animals, breakdown of institutional support, smuggling and inflow of uncontrolled food aids.
- Economic factors- the importance of agriculture to an economy will determine how strict its enforcement of quarantine and control policies will be (FAO, 2001).

2.4 Economic costs of African swine fever

Published literature on the economics of transboundary animal diseases is scarce (Ellis and Putt, 1981). The impacts of these diseases are complex and can extend beyond the direct financial losses associated with an outbreak (Marsh, 1999; Tisdell, Harrison and Ramsay, 1999; McDermott, Randolph and Staal, 1999; Tambi *et al.*, 1999). For ASF in Nigeria, vastly differing financial estimates have been made, ranging from ≈ ₦500,000 (US$3,125) for certain farms in Lagos (Saka, Adesehinwa and Ajala, 2010) to ≈US$ 942,000 (Babalobi *et al.*, 2007). A recent baseline study has indicated that ASF outbreaks in Nigeria may have cost the country approximately ₦4 billion (≈US$25 million) when direct costs, value of dead animals, indirect costs, treatment costs, surveillance costs, intervention costs and costs of burden of the disease are taken into account (Fadiga Jost and Ihedioha, 2011).

Similar evaluations of ASF outbreak costs in other countries have identified amounts of US$1,415,323 for Zambia (Samui *et al.*, 1996), up to US$30 million for Spain (Bech-Nielsen, Bonilla and Sanchez-Viscaino, 1993) and US$5,455 million for the USA (Rendleman and Spinelli, 1999). Very recently, the ASF outbreaks in the

Russian federation which caused the death of about 300,000 pigs are estimated to have cost the country a sum of about US$240 million (Callaway, 2012).

While such animal diseases like ASF have direct financial implications, as outlined above, the social, political, cultural and food security ramifications are often underestimated. Economic costing of animal disease is important to be able to translate disease in economic terms and convince policy makers on the need to invest in the eradication and control of infectious and transboundary animal diseases (FAO, 2001). Analysing the impacts of animal diseases involves risk analysis, cost-benefit analysis and risk acceptability (FAO, 2001). Cost-benefit analysis is a process whereby economic impact(s) is/are weighed objectively against the different management options that exist for a situation using empirical and time-tested values (Rendleman and Spinelli, 1999; Marsh, 1999; Tisdell, Harrison and Ramsay, 1999; Tambi *et al*., 1999).

In view of the financial impacts of outbreaks of ASF and the available options for the control of the disease, it is imperative to perform a benefit-cost assessment of alternative control strategies to manage ASF in the West African countries. These strategies may include stamping out, improvement of biosecurity measures and allowing an ASF disease situation to take its full course without intervention.

2.5 Determination of virulence of ASF

African swine fever presents with different clinical manifestations ranging from peracute and acute to subacute and chronic forms. These clinical forms have been linked to the virulence of the virus, the course of pathogenesis of the disease and the morbidity/mortality accompanying outbreaks in the domestic pig host (Penrith, Thomson and Bastos, 2004). The peracute and acute forms of the disease are often linked to highly virulent virus strains, while the mildly virulent virus strains are responsible for the subacute and sometimes the chronic disease which may also be caused by the low virulent strains. Similarly, in the early stages of a new epidemic or in naïve pig populations, ASF tends to present with a near 100% fatality while this degree of mortality may decline as the disease becomes entrenched in the pig populations.

While a complete understanding of the reasons for the differences in virulence and pathogenicity of the many virus strains is lacking, certain virulence genes have been studied, including the NL-S gene, a herpes simplex virus ICP34.5 gene related

virulence associated gene (Zsak *et al.*, 1996). It is a short form of the 184 amino acid 23-NL gene containing a highly conserved hydrophilic carboxylterminal 56 amino acids (aa). Studies indicate that its deletion in a virus does not affect viral growth in primary macrophage culture, although it did lead to a marked reduction in virulence in pigs and to a significant drop in virus titres (Zsak *et al*., 1996). Similarly, the highly conserved 96 aa UK gene which is upstream of NL-S and is transcriptionally oriented towards the right end of the genome, does not affect the characteristics of ASFV in macrophage culture but results in a marked reduction in virulence in infected pigs and major drop in blood viral titres, when deleted (Zsak *et al*., 1998). Likewise, the single-copy thymidine kinase (TK) gene located in ORF *K196R* and encoding a 196 amino acid polypeptide is partly responsible for viral transcription and DNA synthesis. ASFV virulence in pigs has been known to decrease following deletion of thymidine kinase and results in low multiplicity of infection in swine macrophage *in vitro* and lower virus titres (Moore *et al*., 1998). Since these identified gene segments are known to be partly responsible for the virulence of ASFV, it is likely that key differences in naturally-occurring variants (field strains) may be associated with their degree of pathogenicity. As a first step in assessing this, the present study will focus on the thymidine kinase gene of selected highly and mildly virulent strains as well as those of low virulence, that span a wide geographical and genotypic range.

3.0 SURVEILLANCE FOR AFRICAN SWINE FEVER IN NIGERIA, 2006-2009

Fasina, F. O[1,2*]., Shamaki, D[3]., Makinde, A. A[1]., Lombin, L. H[3]., Lazarus, D. D[1]., Rufai, S. A[1]., Adamu, S. S[1]., Agom, D[1]., Pelayo V[4]., Soler A[4]., Simón A.[4], Adedeji, A. J[1]., Yakubu, M. B[1]., Mantip, S[1]., Benshak, A. J[1]., Okeke, I[1]., Anagor, P[1]., Mandeng, D. C[1]., Akanbi, B. O[1]., Ajibade, A. A[3]., Faramade, I[3]., Kazeem, M. M[3]., Enurah, L. U[3]., Bishop R[5]., Anchuelo, R[5]., Martin, J. H [4] and Gallardo, C[4].

[1]The African swine fever Project Implementation Task Team, National Veterinary Research Institute, Vom, Nigeria

[2]Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, South Africa.

[3]National Veterinary Research Institute, Vom, Plateau State, Nigeria.

[4]Centro de Investigación en Sanidad Animal, CISA-INIA, Valdeolmos, Madrid, Spain.

[5]International Livestock Research Institute, Nairobi, Kenya.

Corresponding author: Dr. Fasina, F. O.; Telephone: +234 7033 12 8949; Email: daydupe2003@yahoo.co.uk

Aim: *To determine the prevalence of African swine fever in Nigeria, estimate the endemicity of infection and generate isolates for use in this project and other works.*

This paper was published in ***Transboundary and Emerging Diseases***, 2010, 57(4): 244-253

Transboundary and Emerging Diseases

Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Surveillance for African Swine Fever in Nigeria, 2006–2009

F. O. Fasina[1,2], D. Shamaki[3], A. A. Makinde[1], L. H. Lombin[3], D. D. Lazarus[1], S. A. Rufai[1], S. S. Adamu[1], D. Agom[1], V. Pelayo[4], A. Soler[4], A Simón[4], A. J Adedeji[1], M. B. Yakubu[1], S. Mantip[1], A. J. Benshak[1], I. Okeke[1], P. Anagor[1], D. C. Mandeng[1], B. O. Akanbi[1], A. A. Ajibade[3], I. Faramade[3], M. M. Kazeem[3], L. U. Enurah[3], R. Bishop[5], R. Anchuelo[5], J. H. Martin[4] and C. Gallardo[4]

[1] The African swine fever Project Implementation Task Team, National Veterinary Research Institute, Vom, Nigeria
[2] Department of Zoology and Entomology, University of Pretoria, Mammal Research Institute, South Africa
[3] National Veterinary Research Institute, Vom, Plateau State, Nigeria
[4] Centro de Investigación en Sanidad Animal, CISA-INIA, Valdeolmos, Madrid, Spain
[5] International Livestock Research Institute, Nairobi, Kenya

Keywords:
ASF; Nigeria; Prevalence; surveillance

Correspondence:
Dr F. O. Fasina. National Veterinary Research Institute, P.M.B. 01, Vom, Plateau State, Nigeria. Tel.: +234 7033 12 8949; Fax: +442 0818 17 080; E-mail: daydupe2003@yahoo.co.uk

Received for publication January 15, 2010

doi:10.1111/j.1865-1682.2010.01142.x

Summary

African swine fever (ASF) has had significant economic and social impact in Nigeria since 1997. However, there has been no effective national response to bring it under control. In this report, we confirm that ASF is still prevalent and widespread in Nigeria. Results from both serosurveillance and virological analyses indicated that ASF is present in most of the agro-ecological zones of the country. Nine per cent (9%) of serum samples and 48% of tissue samples were positive for ASF virus antibody and genome, respectively. Areas with high pig-related activities (marketing, consumption and farming) have higher prevalences compared with areas with less pig activities. Farm-gate buyers, marketing systems and transport of untested pigs within the country assist with the circulation of the virus. Only by putting in place a comprehensive routine surveillance and testing system, reorganizing the market and transportation systems for pigs, implementing on-farm bio-security protocols and considering the option of compensation will it be possible to achieve a significant reduction in ASF prevalence in Nigeria.

Introduction

Pigs play a major role in the socio-economic life of the people of Nigeria. They not only serve as a source of income especially for the rural population but also fulfil an important role in culture and food security. A substantial portion of the country's pig populations is resident in key pig producing, consuming and marketing areas of the country. The country's pig population has risen steadily from about 2 million to over 7 million from 1984 to 1997 (El-Hicheri, 1998). From 1997, widespread outbreaks of African swine fever (ASF) were experienced.

ASF is a haemorrhagic disease of domestic pigs caused by a DNA arbovirus of the genus Asfivirus and family Asfarviridae. The ASF virus (ASFV) has a double-stranded DNA genome, with a variable size of between 170 and 190 kb because of deletions and insertions occurring in the terminal regions of the genome and within a coding gene region of the central region of the genome, termed the central variable region (CVR) (Dixon et al., 2005; Owolodun et al., 2010). All ASF viruses belong to a single serotype. Differentiation is on the basis of genotypes (p72 genotyping and CVR sub-typing), and to date, only one p72 genotype and 6 CVR sub-genotypes have been identified in Nigeria (Bastos et al., 2003, 2004; Rowlands et al., 2008; Owolodun et al., 2010).

Three types of epidemiological cycles of maintenance for ASF virus have been described: a sylvatic cycle that occurs in southern and eastern Africa involving warthogs (*Phacochoerus aethiopicus*) and argasid ticks of the genus Ornithodoros with occasional spill-over to domestic pigs; a cycle in domestic pigs with the involvement of

© 2010 Blackwell Verlag GmbH • Transboundary and Emerging Diseases. 57 (2010) 244–253

3.0.1 Summary

African swine fever (ASF) has had significant economic and social impact in Nigeria since 1997. However, there has been no effective national response to bring it under control. In this report, we confirm that ASF is still prevalent and widespread in Nigeria. Results from both serosurveillance and virological analyses indicated that ASF is present in most of the agro-ecological zones of the country. Nine percent (9%) of serum samples and 48% of tissue samples were positive for ASF virus antibody or genome respectively. Areas with high pig-related activities (marketing, consumption and farming) have higher prevalences compared with areas with less pig activities. Farm-gate buyers, marketing systems and transport of untested pigs within the country assist with the circulation of the virus. Only by putting in place a comprehensive routine surveillance and testing system, reorganizing the market and transportation systems for pigs, implementing on-farm bio-security protocols, and considering the option of compensation will it be possible to achieve a significant reduction of ASF prevalence in Nigeria.

Keywords: ASF; Nigeria; prevalence; surveillance

3.1 Introduction

Pigs play a major role in the socio-economic life of the people of Nigeria. They not only serve as a source of income especially for the rural population but also fulfill an important role in culture and food security. A substantial portion of the country's pig populations is resident in key pig producing, consuming and marketing areas of the country. The country's pig population has risen steadily from about 2 million to over 7 million from 1984 to 1997 (El-Hicheri, 1998). From 1997, widespread outbreaks of African swine fever (ASF) were experienced.

ASF is a haemorrhagic disease of domestic pigs caused by a DNA arbovirus of the genus Asfivirus and family Asfarviridae. The ASF virus (ASFV) has a double-stranded DNA genome, with a variable size of between 170 and 190 kb due to deletions and insertions occurring within the terminal regions of the genome and within a coding gene region within the central region of the genome, termed the central variable region (CVR) (Dixon *et al*.,, 2005; Owolodun *et al*, 2010). All ASF viruses belong to a single serotype. Differentiation is on the basis of genotypes (p72 genotyping and CVR sub-typing) and to date, only one p72 genotype and 6 CVR sub-genotypes has been identified in Nigeria (Bastos *et al.*, 2003; 2004; Rowlands *et al*., 2008, Owolodun *et al*., 2010).

Three types of epidemiological cycle of maintenance for ASF virus have been described: a sylvatic cycle that occurs in southern and eastern Africa involving warthogs (Phacochoerus aethiopicus) and argasid ticks of the genus Ornithodoros with occasional spill-over to domestic pigs, a cycle in domestic pigs with the involvement of Ornithodoros inhabiting pig sties, and a cycle in domestic pigs without the involvement of other hosts or vectors (Bastos et al. 2004). So far only a cycle in domestic pigs, without known involvement of other hosts and vectors, has been implicated in the

endemicity in Nigeria. Although ASF was responsible for large numbers of fatalities amongst the intensively managed and free-range pig populations in Nigeria from September, 1997, the cause of the disease was not confirmed until November, 1997 (El-Hicheri, 1998). Prior to this, the other West African countries including Cote d'Ivoire, Togo, Benin, and Gambia reported outbreaks of ASF and warning signals were sent to other West and Central African countries (El-Hicheri, 1998). Notwithstanding the above, Nigeria remained ill prepared to respond to and control outbreaks of the disease. Consequently, ASF spread rapidly in Nigeria, causing high mortalities during September 1997 and October 1998 (El Hicheri, 1998). An estimated 125,000 pigs from 9 States were reported dead in this initial wave (El Hicheri, 1998). Since the time of this initial epizootic, sporadic outbreaks have persisted in Nigeria with devastating impacts on both subsistence and commercial pig activities (El-Hicheri, 1998; Babalobi *et al*., 2007; Olugasa *et al*., 2007).

To date, despite the widespread losses caused by the epizootics of ASF in Nigeria, and its accompanying socio-economic effects, little progress has been made on the control and eradication strategy (Babalobi *et al*., 2007).

Since there is no vaccine available for ASF, stringent bio-security and prompt diagnosis are the options for an efficient eradication programme for ASF in Nigeria (El Hicheri, 1998). The PCR is an important diagnostic tool for ASF, particularly when animals are dying of acute disease and do not elicit a measurable immune response. In this report, we discuss results of ASF field, serological and virological surveillance in Nigeria, employing visual appraisal and laboratory based techniques respectively.

3.2 Materials and methods

3.2.1. Location

Nigeria lies within the latitude 4^0-14^0N and longitude 2^0-15^0E with a land area of 923,763km^2 and a human population of about 150 million. It is located in West Africa and bounded on the west by Benin Republic, on the north by Niger and Chad, on the east by Cameroon and on the south by the Atlantic Ocean (Figure 3.1). Seventeen (17) states were selected to represent the different agro-ecological zones of the country (Figure 3.2). These agro-ecological zones were aligned within the six geo-political zones of the country for ease of reference (Table 3.1, Appendix C). Stratified sampling with random sampling within clusters of each stratum was used in farm site and slaughter slab/abattoir selections. There is currently no comprehensive database for pig farms in Nigeria and the pig population figure of approximately 9 million is an estimation (Federal Ministry of Agriculture, Abuja, Nigeria, 2007). We marked all major pig producing, consuming and marketing areas by generating multiple random points using the geographic information systems software, and visited such locations; requested the average number of pig herds/farms available from heads of local pig farmers associations, local agricultural officers and extension agents; listed out the numbers and sizes (in ranges, e.g. 1-20 (small), 21-50 (small-medium), 51-100 (medium), >100 (large)); conducted random selections of small, medium and large farms; and carried out random sampling within each selected farm. Not more than five samples were collected per farm. Market and abattoirs were included in the surveillance because they appear to be extensions of farms since no testing is done for pigs before they are transported to the markets/abattoirs. Not more that five samples were collected from an abattoir/market at any point in time. Since it was difficult to determine the actual numbers of pigs per location, the sampling was opportunistic at each location.

A key factor in the selection of sites was to include the main pig producing, pig marketing and pig consuming areas of the country especially areas where there have been previous reports of ASF outbreaks. In certain regions/states of Nigeria cultural and religious factors prevent close association with pigs and these were not included. Thirty-six major towns were visited apart from their surrounding villages and suburbs (Table 3.1).

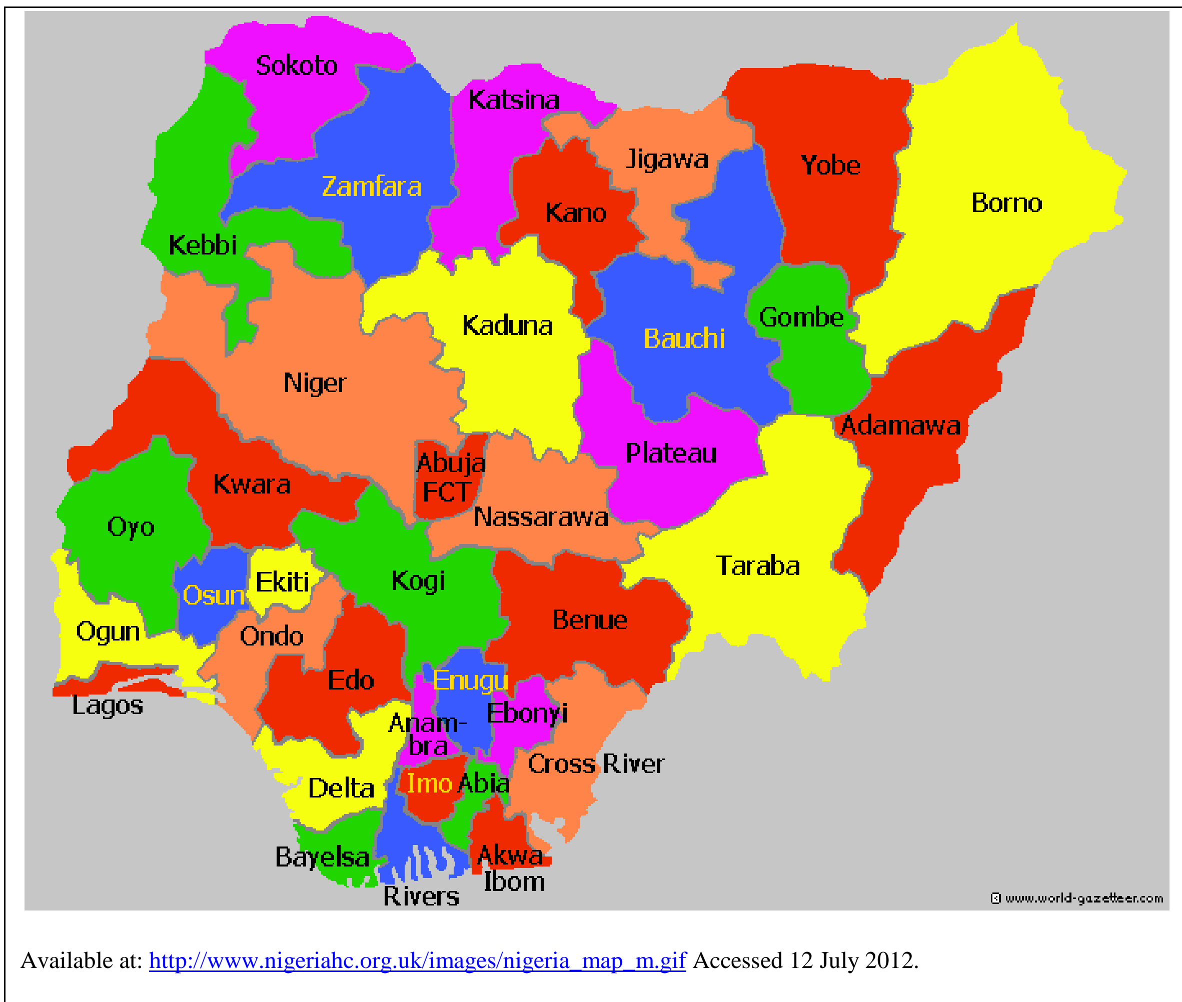
3.2.2. *Sample Collection*

A total of one thousand, two hundred and seventy-six (1276) sera and three hundred and twenty-two (322) groups of tissue samples (894 individual tissues-livers, spleens, lungs, lymph nodes and kidneys) were collected between October 2006 and April 2009 (Table 3.1). The samples were collected from different breeds including crosses of Large White, Landrace, Hampshire and Duroc under free ranged and intensively managed systems. The age of the pigs sampled ranged from three months to approximately four years. In addition, a few local pig breeds mainly from Kebbi and Wukari (≤30) were sampled. While the sera were collected mainly by venipuncture, with some coming directly from collection at the point of slaughter during decapitation, tissues were collected by humane sacrifice (stunning and decapitation) of pigs, which enabled necropsy and tissue collections. All sera and tissues harvested in the field were transported on wet ice (+4^{0}C) and stored at -20^{0}C in the laboratory at the Viral Research Division of the National Veterinary Research Institute (NVRI), Nigeria, until used. All sera and tissues were initially tested in Nigeria by indirect ELISA and PCR, and later sent to the EU Community ASF Reference Laboratory [Centro de Investigación en Sanidad Animal –

Instituto Nacional de Investigación y tecnologia Agraria y Alimentaria, (CISA-INIA)] for confirmatory ASF serological and virological diagnoses.

Figure 3. 1: Political Map of Africa

Available at: http://www.teachervision.fen.com/tv/printables/scottforesman/SSMAP086.pdf Accessed 12 July 2012.



Available at: http://www.nigeriahc.org.uk/images/nigeria_map_m.gif Accessed 12 July 2012.

Geo-political Zones	North West	North Central	North East	South West	South East	South South
States	Kaduna	Benue	Adamawa	Ekiti	Abia	Akwa-Ibom
	Katsina	FCT	Bauchi	Lagos	Anambra	Bayelsa
	Kano	Kogi	Borno	Osun	Ebonyi	Cross-River
	Kebbi	Kwara	Gombe	Ondo	Enugu	Delta
	Sokoto	Nasarawa	Taraba	Oyo	Imo	Edo
	Jigawa	Niger	Yobe	Ogun		Rivers
	Zamfara	Plateau				

Figure 3.2: Map of Nigeria showing the different geopolitical zones and states.

Table 3. 1. ASFV serological and virological resultsof the major pig trading establishments in Nigeria located within 17 states and over 35 localities . Specimen were collected between October 2006 and April, 2009. ELISA and IB were utilised for antibody detection, while PCR and VI were employed for antigen screening and viable virus isolation respectively.

STUDY AREA			ANTIBODY DETECTION					DNA DEMONSTRATION					VIRUS ISOLATION				
REGION	**STATE AND PERIOD OF COLLECTION**	**LOCATION**	**TOTAL**	**POSITIVES**		**NEGATIVES**		**TOTAL**	**POSITIVES**		**NEGATIVES**		**TOTAL**	**POSITIVES**		**NEGATIVES**	
				Nº	***%***	***Nº***	***%***		***Nº***	***%***	***Nº***	***%***		***Nº***	***%***	***Nº***	***%***
South-west	LAGOS (September-October 2008)	OKEARO	10	5	50	5	50	30	29	97	1	3	29	25	86	4	14
		AGEGE	9	0	0	9	100										
		IKORODU	8	0	0	8	100										
		Sub-total	***27***	***5***	***19***	***22***	***81***	***30***	***29***	***97***	***1***	***1***	***29***	***25***	***86***	***4***	***14***
	OGUN (September-November 2008)	IFO	33	0	0	33	100	3	1	33	2	67	1	1	100	0	0
		OWODE EGBA	9	0	0	9	100										
		IJEBU ODE	8	0	0	8	100	5	3	60	2	40	3	1	33	2	67
		Sub-total	***50***	***0***	***0***	***50***	***100***	***8***	***4***	***50***	***4***	***50***	***4***	***2***	***50***	***2***	***50***
	OYO (September 2008- April 2009)	AKINSAWE	14	3	21	11	79										
		BODIJA	134	6	4	128	96	11	4	36	7	64	4	1	25	3	75
		Sub-total	***148***	***9***	***6***	***139***	***94***	***11***	***4***	***36***	***7***	***64***	***4***	***1***	***25***	***3***	***75***
REGIONAL SUBTOTAL			**225**	**14**	**6**	**211**	**94**	**49**	**37**	**76**	**12**	**24**	**41**	**30**	**73**	**11**	**27**
North-east	TARABA (October – December 2008)	USSA	10	0	0	10	100										
		TAKUM	31	5	16	26	84										
		WUKARI	3	0	0	3	100										
		LAU	16	0	0	16	100										
		ZING	17	0	0	17	100										
		Sub-total	***77***	***5***	***6***	***72***	***94***										
	ADAMAWA (October – December, 2008)	YOLA	8	0	0	8	100	2	0	0	2	100					
		NUMAN	42	23	55	19	45	1	0	0	1	100					
		Sub-total	***50***	***23***	***46***	***27***	***54***	***3***	***0***	***0***	***3***	***100***					
	GOMBE (January - April 2009)	GOMBE	62	0	0	62	100										
		Sub-total	***62***	***0***	***0***	***62***	***100***										
REGIONAL SUBTOTAL			**189**	**28**	**15**	**161**	**85**	**3**	**0**	**0**	**3**	**100**	**0**	**0**	**0**	**0**	**0**
South-south	CROSS RIVER (September – November	CALABAR	32	6	19	26	81	19	10	53	9	47	10	4	40	6	60

	2008)																
	AKWA IBOM (September – November 2008)	UYO	16	0	0	16	100										
	EDO (September – November 2008)	BENIN	24	0	0	24	100	32	0	0	32	100					
	DELTA (March – October 2006)	20 LOCATIONS	122	17	14	105	86	5	3	60	2	40	3	1	33	2	67
REGIONAL SUBTOTAL			**194**	**23**	**12**	**171**	**88**	**56**	**13**	**23**	**43**	**77**	**13**	**5**	**38**	**8**	**62**
North-central	PLATEAU (2006-2007, March-December 2008)	JOS 2006-07	54	4	7	50	93	77	36	47	41	53	36	10	28	26	72
		JOS 2008	394	36	9	358	91										
		Pankshin	2	1	50	1	50										
		Sub-total	***450***	***41***	***9***	***409***	***91***	***77***	***36***	***47***	***41***	***53***	***36***	***10***	***28***	***26***	***72***
	BENUE (September – December 2008)	MAKURDI	50	1	2	49	98	70	31	44	39	56	31	7	23	24	77
		GBOKO	25	0	0	25	100	25	20	80	5	20	20	13	65	7	35
		Sub-total	***75***	***1***	***1***	***74***	***99***	***95***	***51***	***54***	***44***	***46***	***51***	***20***	***39***	***31***	***61***
REGIONAL SUBTOTAL			**525**	**41**	**8**	**482**	**92**	**172**	**87**	**51**	**85**	**49**	**87**	**30**	**34**	**57**	**66**
North-west	KADUNA (December 2008 – January 2009)	KADUNA	20	1	5	19	95	19	16	84	3	16	16	14	88	2	13
		CHUKUN	12	0	0	12	100										
		CHIDA	7	0	0	7	100										
		KAFANCHAN	13	1	8	12	92										
		Sub-total	***52***	***2***	***4***	***50***	***96***	***19***	***16***	***84***	***3***	***16***	***16***	***14***	***88***	***2***	***13***
	KEBBI (December 2008)	ZURU	22	0	0	22	100										
REGIONAL SUBTOTAL			**74**	**2**	**3**	**72**	**97**	**19**	**16**	**84**	**3**	**16**	**16**	**14**	**88**	**2**	**13**
South-east	ABIA (October – November 2008)	UMUAHIA	5	0	0	5	100	11	0	0	11	100					
		UMUDIKE	12	0	0	12	100										
		ABA	7	3	43	4	57										
		Sub-total	***24***	***3***	***13***	***21***	***88***	***11***	***0***	***0***	***11***	***100***					
	IMO (October - November 2008)	MBAISE	11	0	0	11	100	5	0	0	5	100					
		OWERRI	10	0	0	10	100										
		Sub-total	***21***	***0***	***0***	***21***	***100***	***5***	***0***	***0***	***5***	***100***					
	ENUGU (October – December, 2008)	ENUGU	4	0	0	4	100	7	0	0	7	100					
		EMENNE	20	0	0	20	100										
		Sub-total	***24***	***0***	***0***	***24***	***100***										
REGIONAL SUBTOTAL			**69**	**3**	**4**	**66**	**96**	**23**	**0**	**0**	**23**	**100**					
NATIONAL TOTAL (2006-2009)			**1274**	**111**	**9**	**1163**	**91**	**322**	**153**	**48**	**169**	**52**	**155**	**79**	**50**	**76**	**50**

3.2.3. ASF antibody detection

3.2.3.1. Indirect enzyme-linked immunosorbent assay OIE –ELISA.

The details of the test are fully described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees) (2008). Briefly, ELISA microtiter plates (Nunc Polysorp® Immunoplates) were incubated at 4ºC overnight with 100 µl/well of ASFV soluble cytoplasmic antigen at a previously determined optimal concentration, in coating buffer (0.1 M carbonate buffer pH 9,6). The coated plates were washed with PBS-pH 7.5 containing 0.05% (v/v) Tween 20 (PBS-T) and used immediately or stored at -20ºC until use. Porcine sera were added to the plates at a 1:30 dilution in PBS-T and incubated for 1h at 37ºC. Reference sera were included on each plate. Horse Radish Peroxidase (HRP)-labeled protein A (HRP-Protein A, PIERCE) was diluted 1:5000 in PBS-T and added to the plates and incubated for 1 h at 37ºC. After washing the plates, 0.2 ml of 3-Dimethylaminobenzoic acid+3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (DMBA-MBTH) substrate (Sigma) were added per well. The reactions were stopped by the addition of 50 µl of 3N H_2SO_4, and the optical density (OD) was measured at 620 nm using a spectrophotometer Multiskan EX® (Thermos Scientific, Vantaa, Finland) after incubation for 10 minutes at room temperature.

The cut-off was determined using the following formula: [Optical density negative serum X 1] + [Optical density positive serum X 0.2].

3.2.3.2. Immunobloting

Sera yielding positive and doubtful results on ELISA were retested using the OIE (2008) recommended confirmatory test, the immunoblotting (IB) assay. Briefly, ASF vp30 blotted nitrocellulose strips were incubated by complete immersion in separate partitions in a plastic trough with 2% (w/v) blocking buffer (non-fat-dried-milk in PBS; pH 7.2), for 30minutes with continuous agitation (Gallardo and Arias, 2009). The blocking buffer was discarded and fresh blocking buffer was added to 0.5ml of the test and control sera to make dilutions of 1/40. This mixture was incubated for 45minutes at 37^0C, with continuous agitation. The content was discarded and the single strips and wells were thoroughly washed four times in blocking buffer. A 1/1000 dilution of HRP-Protein A conjugate was added (0.5 ml) and the content was incubated for 45 minutes at 37^0C, with continuous agitation. The washing steps were repeated and 0.5ml of the substrate (4-Chloronaphtol/methanol) solution was added to each well of the trough. The reaction was stopped after 15 minutes by running cold water over the strips. Sera were regarded as positive only if the strips they were incubated with displayed bands of identical size and position as those that reacted with the positive control sera.

3.2.4. ASF virological detection

3.2.4.1. Nucleic acid extraction and genomic DNA amplification

DNA was extracted directly from a 10% saline buffer suspension of each of the 322 pooled tissues using a nucleic acid extraction kit (Nucleospin/ Machery-Nagel–Cultek) following the manufacturers procedures. A PCR assay using the ASF diagnostic primers

PPA1 [5´-AGTTATGGGAAACCCGACCC-3´], PPA2 [5´- CCCTGAATCGGAGCATCCT-3´] which generate an amplicon of 257 bp within the p72 gene (Aguero et al., 2003) was used to confirm the presence of ASFV DNA. For the amplification of DNA, 23µl of the previously prepared PCR mix (Roche) was added to 2µl of the DNA template in a 0.2ml reaction tube. A similar preparation was made for the positive (Spain '70) and negative controls (nuclease free distilled water) templates. A DNA marker was added to one lane on each side of the gel. The reaction mixture was treated as follows: (i) incubated for 10 min at 95°C; (ii) subjected to 40 cycles of PCR, with 1 cycle consisting of 15 s at 95°C, 30 s at 62°C, and 30 s at 72°C; and (iii) incubated for 7 min at 72°C. The PCR products were analysed by electrophoresis through 2% agarose gel and visualisation under UV light. The results were only taken as valid if all the negative controls showed no bands while the positive controls displayed bands at the 257bp region of the DNA marker.

3.2.4.2. Virus isolation

Antibody negative/naïve pigs were used for the preparation of primary leukocyte cultures (PLC) as previously described (Malquimst and Hay, 1960). These PLC were used for the isolation of viruses from ASF antigen positive samples. Briefly, cells were seeded into 96-well tissue culture grade microtitre plates (200 µl; 300,000 cells per well) in homologous swine serum, and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. Three day cultures were infected by quadriplicates of a 10% suspension of PCR positive ground tissues supplemented with 5 µg/ml gentamycine sulphate (BioWhittaker) and incubated for 24 hours at 37ºC. After inoculation, a 20µl preparation of 1% homologous Red Blood Cells (RBC) in buffered saline was added to each well. The

plates were examined for haemadsorption and cytopathic effects over a 6 day period. The negative samples were blind passaged three times.

3.2.5 Statistical Analysis

Analyses were performed using StatGraphics v2.0 and data were entered on the nominal scale. Pearson Chi square (χ^2) tests were used to determine the associations between the results of the laboratory tests and the regions/locations where the samples originated. Antibody detections and DNA demonstrations were initially cross-tabulated separately with the regions/locations of samples. Virus isolations results were excluded from similar statistics due to wide disparities in data available from the different regions. The percentage distributions of the test results relative to the regions and locations were generated and the Contingency Coefficient was then used to measure the strength of the association, if any. The results were exported into Microsoft Excel® worksheet and rounded off to the nearest whole number.

3.3 Results

3.3.1 Serology

One thousand, two hundred and seventy six serum samples were classified as positive or negative by indirect ELISA followed by confirmatory immunoblotting. From the sera analysed, 111 (9%) tested ASF antibody positive and 1163 (91%) yielded negative results. Specifically, 189 sera tested positive for ASF antibody by indirect ELISA but only 111 were confirmed by immunoblotting assay. The remaining 78

ambiguous/doubtful sera which were spread across geographical regions were negative by immunoblotting assay. The regional prevalence to ASF antibodies varied as follows: South-west (6%), North-east (15%), South-south (12%), North-central (8%), North-west (3%) and South-east (4%). At $P<0.05$, the χ^2 value was 18.232 and Contingency Coefficient was 0.2 (Figure 3.3a).

3.3.2 DNA Demonstration and Virus Isolation

PCR was performed on 322 organ pools from domestic pigs. Following, amplification of the pooled samples, a single major amplicon of approximately 260 bp was generated from 153 (48%) of the samples (Figure 3.3b). The specificity of the amplicons obtained was confirmed by *BsmaI* restriction analyses previously described by Aguero *et al.*, (2003). The regional prevalence to ASFV genome were as follows: South-west (76%), North-east (0%), South-south (23%), North-central (51%), North-west (84%) and South-east (0%) ($P<0.05$; χ^2 value = 63.844; Contingency Coefficient = 0.2.

All samples which tested positive on PCR underwent virus isolation in pig leucocyte cultures since the OIE regards the test as a gold standard in ASF diagnosis. From the 155 PCR positive samples, only 79 (50%) yielded positive virus isolation results. Only haemadsorbing ASFV were isolated.

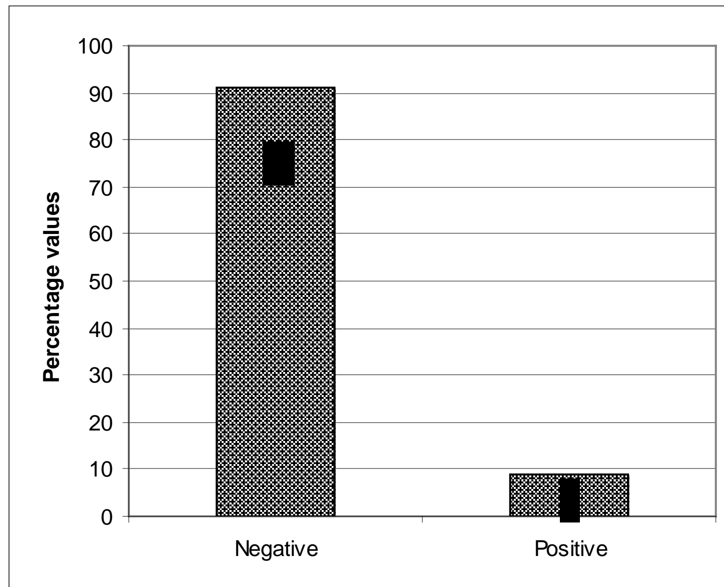


Figure 3. 3a

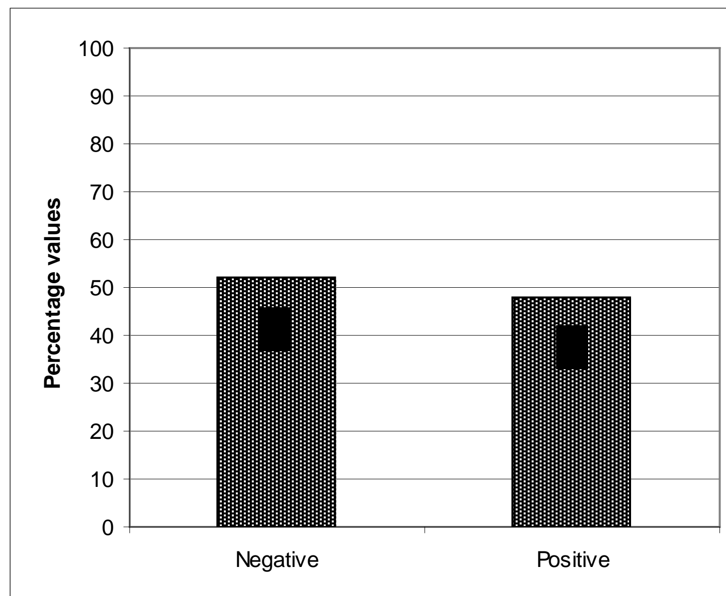
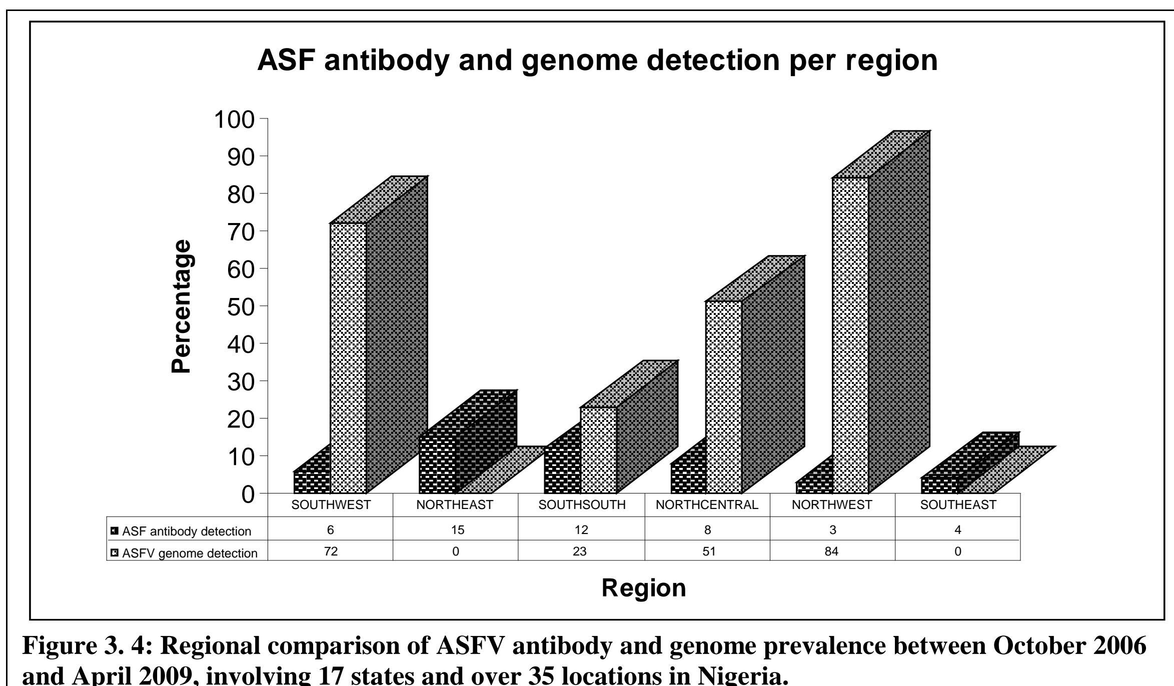


Figure 3. 3b

Figure 3. 3a & b: Comparative ASFV antibody (a) and genome detection (b) in samples collected from selected states in Nigeria between October 2006 and April 2009.



	SOUTHWEST	NORTHEAST	SOUTHSOUTH	NORTHCENTRAL	NORTHWEST	SOUTHEAST
ASF antibody detection	6	15	12	8	3	4
ASFV genome detection	72	0	23	51	84	0

Figure 3. 4: Regional comparison of ASFV antibody and genome prevalence between October 2006 and April 2009, involving 17 states and over 35 locations in Nigeria.

Combined serological and virological ASF diagnostic results are summarized in Table 3.1, and indicate the prevalence of ASF in target Nigerian states. Certain patterns were observed in the prevalence of ASF using the serological and virological analyses. Oke-Aro in Lagos State, a facility that has a holding capacity of up to 300,000 pigs and many pig abattoirs, presented with high seroprevalence (50%) (ELISA and immunoblotting) and virus prevalence, as shown by both PCR and virus isolation (86%) results. In Numan in Adamawa State, a major pig trading centre, the ASF surveillance also showed high seroprevalence of ASF (55%) although insufficient tissues were collected to perform a comprehensive analysis including PCR and VI. In contrast, in Gboko in Benue state and Kaduna city (Kaduna State), a higher prevalence of ASF virus was observed (65% and 88% respectively) compared with positive serological results (0% and 5%) However, in the course of the surveillance, it was impossible to obtain significant numbers of sera from Kafanchan International pig market, another important pig marketing area, and

therefore results from Kafanchan (Kaduna State) may be biased when compared to the overall results.

Similarly, the following locations had evidence of significant virus activity based on PCR results: Ijebu-Ode, Ogun State 3/5 (60%); Delta, Delta State 3/5 (60%); Calabar, Cross-River 10/19 (53%); Jos, Plateau State 36/77 (47%); Makurdi, Benue State 31/70 (44%) and Bodija, Oyo State 4/11 (36%) (Figure 3.1). Figure 3.3 shows comparative serological and PCR regional results indicating the higher virus prevalence in North and South Western regions in contrast with zero percentage (0%) prevalences recorded from the South and North eastern parts of the country.

3.4 Discussion

The results show that ASF is still a problem in the Nigerian pig industry. We are aware that our surveillance system is subject to certain limitations. The surveillance was carried out in selected locations and some regions may have been under represented based on the estimated pig population as verbally communicated by stakeholders. The sampling targeted areas of high pig concentrations because the distribution of pigs varies due to religious and cultural differences in human population. Within the target areas, the sampling was startified and randomised as described above. Similarly, sample size was not as good in some areas as others because we depended largely on the cooperation of ordinary farmers, farmer groups, assistance from the State, Local Government Authorities and other relevant bodies to carry out surveillance in the field. Some farmers expressed dissatisfaction with the government and did not allow sampling in their farms since no compensation was paid for pigs that had died due to ASF.

Although no stamping out was carried out, farmers were of the opinion that outbreaks should have been controlled by culling and conpensation paid as was the case in the control of HPAI H5N1. The surveillance results reported here represent the most comprehensive study on the ASF situation in the West African region to date despite reports from other investigators (Babalobi *et al*., 2007; Luther *et al*., 2007; Olugasa, 2007; Olugasa *et al*., 2007 and Owolodun *et al*., 2007).

The tissue samples were largely collected from the abattoirs, submitted to the central laboratory from farms or through direct purchase of live pigs from farms followed by humane slaughtering. The fact that the tissue samples yielded more positive results than sera could be attributed to farmers' practices: during an active outbreak, farmers often will not report to authorities but will rapidly seel off pigs before they die of ASF; furthermore unthrifty and sick animals are culled first for slaughter. This may also be due to the fact that animals were culled early in sickness or during the per-acute phase before a measurable humoral immune response can be generated. Since PCR test detects only a fragment of genome sequence of the virus, the PCR may be positive, even when no infectious virus is detected by virus isolation suggesting the possibility that some of the pigs may no longer be infectious.

Fasina *et al*., (2009) had previously reported higher seroprevalences around the abattoir/market areas when compared with farms. Since pigs introduced to the markets/abattoirs rarely stay for more that three weeks before slaughter, it may be possible that antibodies are developed while still on farms, suggesting infection by mild strains of ASFV which permit the pigs to live longer and develop detectable antibodies. Most of the tissue samples used in this study came from the submitted samples, slaughter

slabs, abattoir and meat shops, although a deliberate effort was made to buy whole animals from farms and slaughter these for tissue sample collection. The tissue samples from Delta State were collected during an active ASF outbreak, which lasted for about seven months (March-October, 2006 and this may be responsible for the higher antigen prevalence than others states within the region. The overall results indicate that a mild form of ASF virus may also be circulating in the field, resulting in a high number of persistently infected pigs. This may be particularly true in regions where both serological and virological results indicated high values. A similar report was recently published by Owolodun and colleagues (2010). In addition, it is highly likely that the difference between serologic and virologic results are linked to pigs that have better innate resistance to the pathogenic effects of the virus and recover completely. Penrith et al. (2004), had earlier described such resistance in an endemic pig population in South Africa.

Lower prevalences were recorded from the South-eastern and South-southern States probably because these regions are not involved in trading pigs with other regions of the country, as reported by the Eastern Nigerian farmers interviewed. A similar situation was obtained from the Zuru area of Kebbi State (North-west) and extremes of Taraba State (North-east), although Calabar, Cross River State (South-south) presented with a higher prevalence. It was, however, discovered that most of the pigs slaughtered in Calabar originate from Numan, Adamawa State (North-east) and parts of Nasarawa State (North-central).

From these analyses, it was suggested that the sale and circulation of infected animals is an important factor in keeping ASF virus in curculation. Other contributing factors are

visits of farm-gate-buyers who move from farm to farm, lack of compensation for compulsory slaughter in outbreak situations, lack of awareness by farmers and lack of/poor implementation of bio-security (El-Hicheri, 1998).

It is important to put in place a comprehensive routine surveillance and testing system, reorganize the market and transportation systems for pigs and consider the option of compensation for compulsory slaughter in outbreak situations to achieve a reduction of new ASF infections in Nigeria.

Finally, this surveillance has provided valuable base-line data on the probable role of trade movements in the epidemiology of ASF in Nigeria. It will however be important to consider the roles of husbandry systems (free-ranging/scavenging versus confined), swill feeding, possible role of wild suids and other factors that may contribute to the epidemiology of ASF in Nigeria.

Acknowledgement

The ASF Project Implementation Task Team wish to thank the Executive Secretary, Agricultural Research Council of Nigeria, Abuja for funding this project under the Project Code 025060410100000 (*Development of rapid and effective Diagnostic and Control tools for African Swine Fever*). We wish to appreciate the Executive Director, National Veterinary Research Institute for permission to publish our findings. We are grateful to all the technical staff that contributed to the success of this work. International Livestock Research Institute, Kenya; Centro de Investigación en Sanidad Animal, CISA-

INIA, Madrid, Spain and European Union ASF Risk Project are acknowledged for their financial and technical assistance.

3.5 References

Agüero, M., J. Frenandez, L. Romero, C. Sanchez-Mascaraque, M. Arias, and J. M. Sanchez-Viscaino, 2003: Highly sensitive PCR assay for routine diagnosis of African swine fever virus in clinical samples. *J Clin Microbiol* 41 (9), 4431-4434.

Babalobi, O.O., B.O. Olugasa, D.O. Oluwayelo, I.F. Ijagbone, G.O. Ayoade, S.A. Agbede, 2007: Analysis and evaluation of mortality losses of the 2001 African swine fever outbreak, Ibadan, Nigeria. *Trop. Anim. Hlth Prod* 39, 533-542.

Bastos, A.D.S., M.-L. Penrith, C. Crucie`re, J. Edrich, G. Hutchings, F. Roger, E. Couacy-Hymann, and G.R. Thomson, 2003:. Genotyping field strains of African swine fever virus by partial *p72* gene characterization. *Archives of Virology* 148: 693–706.

Bastos, A.D.S., M.-L. Penrith, F. Macome, F. Pinto, G.R. Thomson, 2004: Co-circulation of two genetically distinct viruses in an outbreak of African swine fever in Mozambique: no evidence for individual co-infection. *Vet. Microbiol.* 103, 169–182

Dixon, L. K., J. M. Escribano, C. Martins, D. L. Rock, M. L. Salas, and P. J. Wilkinson, 2005: Asfarviridae. In: Fauquet, C. M., M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (eds), Virus Taxonomy, VIIIth Report of the International Committee on Taxonomy of Viruses, pp. 135–143. Elsevier Academic Press, London

El-Hicheri K., 1998: Emergency Assistance on control and eradication of an outbreak of African swine fever in Western Nigeria. *Report of the FAO Consultancy Mission to Nigeria.* TCP/NIR/7822(E). FAO, Rome, December, 1998.

Fasina F. O., D. Shamaki, A. A. Makinde, L. H. Lombin and the ASF PITT, 2009: Field surveillance and laboratory diagnoses of African swine fever in Nigeria.

International Symposium on Sustainable Improvement of Animal Production and Health. Vienna Austria, IAEA-CN-174-151. Pp300-302. Also available at: http://www-naweb.iaea.org/nafa/aph/BookOfExtendedSynopses.pdf

Gallardo, C. and M. Arias, 2009: *ASF Diagnosis Protocols*. CISA-INIA, Madrid, Spain

Luther, N.J., K.A. Majiyagbe, C.I. Nwosuh, D. Shamaki, 2007a: The epizootiology, prevalence and economic aspects of African swine fever in parts of Middle Belt central states of Nigeria. *Trop Vet* 25, 96-100.

Luther, N.J., D. Shamaki, K.A.Majiyagbe, C.I. Nwosuh, 2007b: The prevalence of African swine fever in parts of the middle belt central states of Nigeria. *Bulletin Anim Prod Hlth in Africa* 55, 79-81.

Malmquist, W. and D. Hay, 1960: Haemadsorption and cythopathic effect produced by African swine fever virus in bone marrow and buffy coat cultures. *American J Vet Res* 21, 104-108.

Olugasa, B.O. 2007. Serological evidence of African swine fever virus infection in commercial pig herds in Southwest Nigeria. *African J Livestock Extension* 5, 61-66.

Olugasa, B.O., I.F. Ijagbone, P. Calistri, A. Conte, J. E. Freier, M. P. Ward, 2007. Pattern of spread of African swine fever in south-western Nigeria, 1997-2005. *Veterinaria italiana* 43, 621-628.

Owolodun, O.A., B. Yakubu, J.F. Antiabong, O.K. Adefalujo, M.E. Ogedengbe, E.T. Obisakin, D. Shamaki, 2007: Molecular assessment of African swine fever in North-Central Nigeria. *Bulletin Anim Prod Hlth in Africa,* 55, 96-103.

Owolodun, O. A., A. D. S. Bastos, J. F. Antiabong, M. E. Ogedengbe, P. S. Ekong, B Yakubu,. 2010: Molecular characterisation of African swine fever viruses from Nigeria (2003–2006) recovers multiple virus variants and reaffirms CVR epidemiological utility. *Virus Genes,* DOI 10.1007/s11262-009-0444-0. Published online 6th January, 2010.

Penrith, M. L., G. R. Thomson, A. D. Bastos, O. C. Phiri, B. A. Lubisi, E. C. Du Plessis, F. Macome, F. Pinto, B. Botha and J. Esterhuysen, 2004: An investigation into natural resistance to African swine fever in domestic pigs from an endemic area of South Africa. *Revue sci Tech Off Int Epizooties* 23, 665-677.

Rowlands, R. J., V. Michaud, L. Heath, G. Hutchings, C. Oura, W. Vosloo, R. Dwarka, T. Onashvili, E. Albina, and L. K. Dixon, 2008: African swine fever virus isolate, Georgia, 2007. *Emerg. Infect. Dis*. 14, 1870–1874.

World Organisation for Animal Health (OIE), 2008: African swine fever. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees). Chapter 2.8.1; Web (Sixth) edition. Available at http://www.oie.int/eng/normes/mmanual/2008/pdf/2.08.01_ASF.pdf . Accessed on 20th January, 2009.

4.0 COST IMPLICATIONS OF AFRICAN SWINE FEVER IN SMALLHOLDER FARROW-TO-FINISH UNITS: ECONOMIC BENEFITS OF DISEASE PREVENTION THROUGH BIOSECURITY

Fasina, F. O[1, 2]. Lazarus, D. D[3]., Spencer, B. T[1]., Makinde, A. A[3]. and Bastos, A. D. S[2].

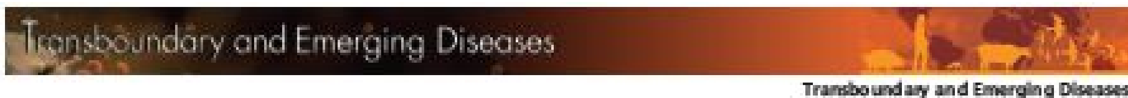
1 Section of Pig Health, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa.

2 Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0028, Pretoria, South Africa.

3 African swine fever Project Implementation Task Team, National Veterinary Research Institute, Vom, Plateau State, Nigeria.

Aim: *To determine the economic losses associated with African swine fever in smallholder farms, and quantify the cost of alternative control (biosecurity) to justify its use as a control mechanism.*

This paper was published online in ***Transboundary and Emerging Diseases***, 2012, 59(3): 244-255, DOI: 10.1111/j.1865-1682.2011.01261.x



Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Cost Implications of African Swine Fever in Smallholder Farrow-to-Finish Units: Economic Benefits of Disease Prevention Through Biosecurity

F. O. Fasina[1,2], D. D. Lazarus[3], B. T. Spencer[1], A. A. Makinde[3] and A. D. S. Bastos[2]

[1] Section of Pig Health, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[2] Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Hatfield, Pretoria, South Africa

[3] African swine fever Project Implementation Task Team, National Veterinary Research Institute, Vom, Plateau State, Nigeria

Keywords:
African swine fever, biosecurity, pigs, benefit-cost analysis

Correspondence:
F. O. Fasina, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa.
Tel.: +27 12 529 8069;
Fax: +27 12 529 8396;
E-mail: daydupe2003@yahoo.co.uk

Received for publication June 24, 2011

doi:10.1111/j.1865-1682.2011.01261.x

Summary

African swine fever remains the greatest limitation to the development of the pig industry in Africa, and parts of Asia and Europe. It is especially important in West and Central African countries where the disease has become endemic. Biosecurity is the implementation of a set of measures that reduce the risk of infection through segregation, cleaning and disinfection. Using a 122-sow piggery unit, a financial model and costing were used to estimate the economic benefits of effective biosecurity against African swine fever. The outcomes suggest that pig production is a profitable venture that can generate a profit of approximately US$109 637.40 per annum and that an outbreak of African swine fever (ASF) has the potential to cause losses of up to US$910 836.70 in a single year. The implementation of biosecurity and its effective monitoring can prevent losses owing to ASF and is calculated to give a benefit-cost ratio of 29. A full implementation of biosecurity will result in a 9.70% reduction in total annual profit, but is justified in view of the substantial costs incurred in the event of an ASF outbreak. Biosecurity implementation is robust and capable of withstanding changes in input costs including moderate feed price increases, higher management costs and marginal reductions in total outputs. It is concluded that biosecurity is a key to successful pig production in an endemic situation.

Introduction

African swine fever virus infected the Nigerian pig population in 1997 with heavy mortalities and huge economic/social impacts (El-Hicheri, 1998). Both the subsistence and commercial pig raising activities were severely disrupted by these infections. The virus continues to circulate causing sporadic or sometimes sustained infections in Nigerian pigs with further significant impacts on the swine industry (Babalobi et al., 2007; Fasina et al., 2010). Possible reasons for the persistence of the virus in the Nigerian pig population include continuous presence of persistently infected and carrier sero-positive pigs on farms, uncontrolled breeding programmes, pig product movements, traders and middlemen operations and most importantly the lack of or poorly implemented biosecurity measures (Olugasa and Ijagbone, 2007).

Field observations and opinion polls of some 95 smallholder pig farmers (Mean herd size = 71, Range = 5–450 pigs; 1st quartile = 17; 3rd quartile = 84) in parts of Nigeria revealed a huge deficiency in the understanding and implementation of basic concepts of biosecurity at farm level. These farmers despite reporting implementation of principles of hygiene in their farms and having experienced shared infections continued to keep/sell survivor pigs, slaughtered infected pigs on their premises, visited infected premises and slaughter slabs within the communities without taking precautionary measures,

© 2011 Blackwell Verlag GmbH • Transboundary and Emerging Diseases. 59 (2012) 244–255

4.0.1 Summary

African swine fever remains the greatest limitation to the development of the pig industry in Africa, and parts of Asia and Europe. It is especially important in West and Central African countries where the disease has become endemic. Biosecurity is the implementation of a set of measures that reduce the risk of infection through segregation, cleaning and disinfection. Using a 122-sow piggery unit, a financial model and costing were used to estimate the economic benefits of effective biosecurity against African swine fever. The outcomes suggests that pig production is a profitable venture that can generate a profit of approximately US$109,637.40 per annum and that an outbreak of African swine fever (ASF) has the potential to cause losses of up to US$910,836.70 in a single year. The implementation of biosecurity and its effective monitoring can prevent losses due to ASF and is calculated to give a benefit cost ratio of 29. A full implementation of biosecurity will result in a 9.70% reduction in total annual profit, but is justified in view of the substantial costs incurred in the event of an ASF outbreak. Biosecurity implementation is robust and capable of withstanding changes in input costs including moderate feed price increases, higher management costs and marginal reductions in total outputs. It is concluded that biosecurity is a key to successful pig production in an endemic situation.

Keywords: African swine fever, Biosecurity, pigs, benefit-cost analysis.

4.1 Introduction

African swine fever virus infected the Nigerian pig population in 1997 with heavy mortalities and huge economic/social impacts (El-Hicheri, 1998). Both the subsistence and commercial pig raising activities were severely disrupted by these infections. The virus continues to circulate causing sporadic or sometimes sustained infections in Nigerian pigs with further significant impacts on the swine industry (Babalobi, *et al*., 2007; Fasina *et al*., 2010). Possible reasons for the persistence of the virus in the Nigerian pig population include continuous presence of persistently-infected and carrier sero-positive pigs on farms, uncontrolled breeding programmes, pig product movements, traders and middlemen operations and most importantly the lack of or poorly implemented biosecurity measures (Olugasa and Ijagbone, 2007).

Field observations and opinion polls of some 95 smallholder pig farmers [Mean herd size =71, Range = 5-450 pigs; 1st quartile = 17; 3rd quartile = 84] in parts of Nigeria revealed a very large deficiency in the understanding and implementation of basic concepts of biosecurity at farm level. These farmers despite reporting implementation of principles of hygiene in their farms and having experienced shared infections continued to keep/sell survivor pigs, slaughtered infected pigs on their premises, visited infected premises and slaughter slabs within the communities without taking precautionary measures, sometimes shared equipment, permitted the entrance of farm-gate buyers into the farm premises and did not make an effort to ascertain the ASF status in their immediate community.

Profitability (the excess of incomes over expenses) remains the principal driver for involvement in pig rearing, hence the understanding of this factor and its use in the introduction and maintenance of principles of biosecurity at farm level becomes important for controlling ASF at farm level, most especially in the small to medium scale piggeries and farming communities.

Furthermore, for the farmers to take a decision to implement and provide sustained support for disease management programs like ASF, financial considerations (profit and or benefit arising from such a decision) is often important, in addition to the following factors:

- Additional workload on the workers and whether this can be adopted easily into the current farm operations
- Complexities in changes in management procedures due to the new protocols
- Requirements for increased levels of investment
- Cheaper alternatives that achieve the same proposed solution (biosecure environment)
- Availability of funds and means to implement the proposed measures
- Commitment of staff including necessary training, understanding of the risk of infection and its severity,
- Incentives to offset the burden placed on workers to ensure proper implementation and secure their involvement
- Cost of compliance and monitoring following implementation
- Laws and regulations that permit or negate such proposed intervention

Based on the above, an understanding of total cost determination is the critical starting point for any positive intervention to be implemented at farm level. For any intervention to survive the keen competitive environment of limited resources, the value or benefit must be clear and the application easy and suited to the local conditions.

The use of economics has been advocated as an effective tool in the management of transboundary animal diseases and a previous study has demonstrated that the understanding of economics of animal diseases including the management and intervention options at herd-level remains a key strategy for rapid implementation of animal disease control (Marsh, 1999; FAO, 2001). Babalobi *et al*. (2007) previously investigated the cost implications of ASF outbreaks in south-west Nigeria using a cost analysis, but to date, no farm level study has been conducted to determine the impact and benefits of intervention in smallholder operations. Benefit-cost analysis represents the most comprehensive standard

financial analysis to evaluate animal disease situations with positive outcomes (Marsh, 1999; Tambi *et al*., 1999; Tisdell, 1999; FAO, 2001).

The aim of this study was to estimate the profitability of biosecurity implementation using a 122-sow farrow-to-finish pig farm model to determine the benefit-cost effects of required interventions. The results are expected to serve as basic guidelines to assist farmers in measuring profitability of farm operations so that they can make informed decisions regarding biosecurity implementation in against the backdrop ofmany competing financial interests.

4.2 Materials and method

4.2.1 Choice of farms

Selected farms were visited and evaluated including small-scale operators (<100-sow units), medium-scale operators (101 to 250-sow units) and large-scale operators (>250-sow units) to compare and contrast production parameters and industry standards. Standardized production data were collected using available literature (Carr, 1998; Stalder *et al*., 2011) and farm data were collated by personal interviews with farmers and careful examination of farm records. Farm data were further confirmed with pig farm consultants and assessed against published data to check for consistency. Critical values where farmers/consultants need to intervene to ensure optimum productivity were also determined and are presented in Table 4.1. Based on economic feasibility and detailed records of farm operations, a 122-sow farrow-to-finish unit producing porkers (70kg liveweight pigs) was ultimately selected for the model.

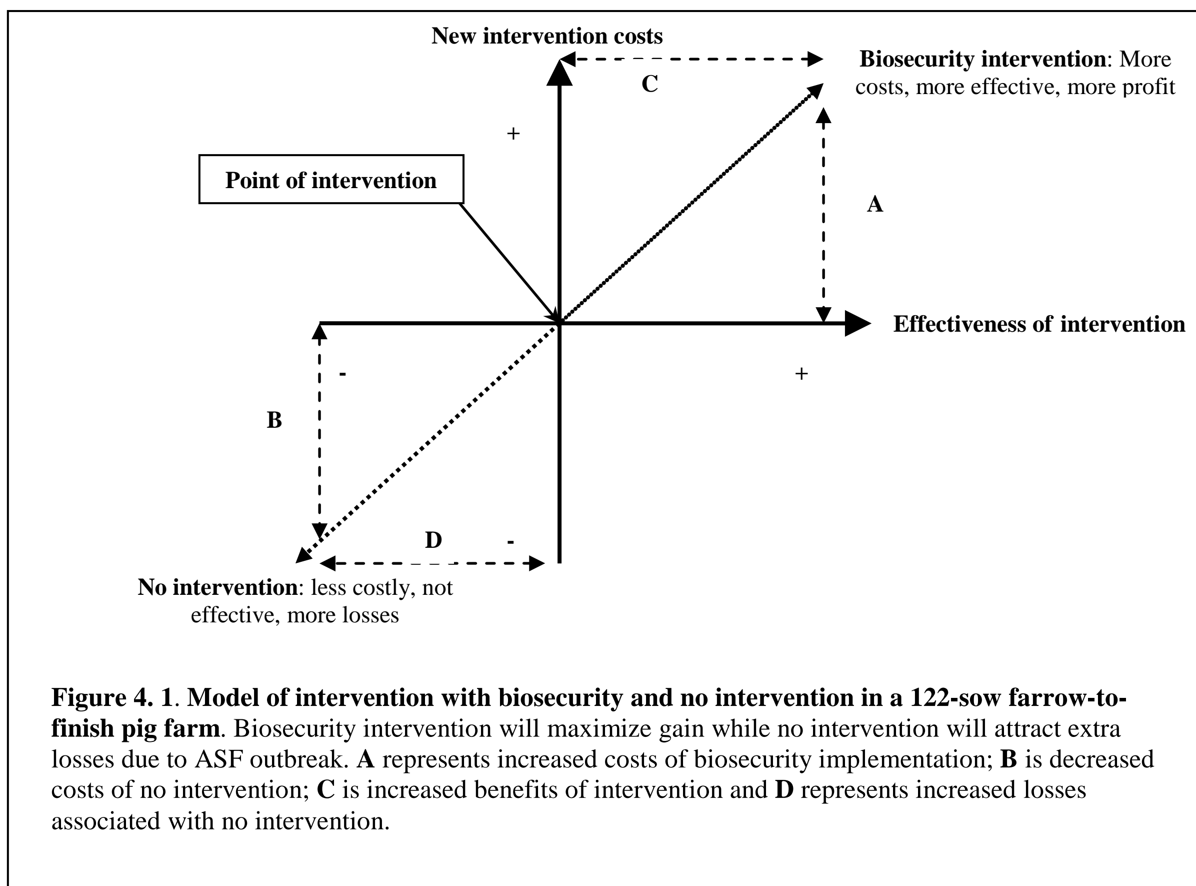


Figure 4. 1. **Model of intervention with biosecurity and no intervention in a 122-sow farrow-to-finish pig farm**. Biosecurity intervention will maximize gain while no intervention will attract extra losses due to ASF outbreak. **A** represents increased costs of biosecurity implementation; **B** is decreased costs of no intervention; **C** is increased benefits of intervention and **D** represents increased losses associated with no intervention.

4.2.2 Identification of risk factors associated with African swine fever virus introduction

When considering possible means of disease introduction, the following were identified as potential routes for African swine fever virus introduction:

- Soft-shelled eyeless ticks (*Ornithodoros moubata* complex) present in the community. This vector has never been implicated in the outbreaks of ASF in West Africa. However, a recent study indicates the presence of an *Ornithodoros* species that may play a role in the epidemiology of ASF in West Africa (Vial *et al*., 2007).
- Warthogs, bushpigs, red-river hogs presence. All three wild suid species occur in the West African sub-region (Jori & Bastos 2009). Contact with domestic pigs kept under intensive management is, however, unlikely.
- Infected pigs present in the farm
- Infected or in-contact pigs bought into the farm without quarantine

- Infected service boars used for natural mating or AI
- Mixing of pigs from different origins and exposures including those from farms, agricultural shows, markets and those returned to the farm through no sale.
- Feeding of raw swill, infected meat scraps from homes or from international carriers (air, water)
- Exchanging of feed bags at the feed mills
- Farm workers raising pigs at home
- Farm workers and managers consulting for other farms
- Farm workers and managers visiting pig abattoirs especially with farm clothes and boots.
- Input suppliers / marketing personnel visiting multiple farms/location/villages/herds per day
- Farm gate buyers visiting several farms/herds/markets to select animals to buy
- Animal health workers and veterinarians consulting for several farms per day
- Contaminated vehicles used to transport input supplies and feed
- Contaminated vehicles used to transport pork and other abattoir waste
- Contaminated farm equipment and implements being used at multiple farms / sites
- Improper disposal of pig by-products including manure and slurry, intestinal content, abattoir waste and blood
- Free-range/scavenging pigs in contact with farms.
- Inadequate access to quality veterinary services and advice.
- Biosecurity lapses in cleaning, disinfection and decontamination.
- Lack of compensation for culled animals.

Based on the above ASF infection risk factors for a 122-sow farrow-to-finish pig farm, the biosecurity measures considered in this study were included to ensure maintenance of a closed herds, easy integration into current farming practices, enhanced farm operations that instill pride in workers, certification to ensure that animals entering and leaving the farm are free of infection, prevention of direct contact with possible infected sources (ticks, wild

pigs and free-range pigs), prevention of indirect contact (formites, tools, tyres etc), prevention of within-farm (pen-to-pen) and inter-farm/inter-site spread of infection

Specifically, the following biosecurity measures were considered to be applicable to the pig farms:

Segregation: Erection of fence and gate, control and monitoring of physical barrier, enforcement of change of footwear and clothing, and restricting the entry of vehicles or dipping of tyres in case of necessary entrance.

Cleaning: Daily sweeping, routine washing of the pen with copious amount of water, thorough washing with soap, water and brush to ensure that no visible dirt is seen on the surface of building and materials, dry cleaning of all material that are not water-resistant.

Disinfection: Usage of appropriate disinfectant to sanitize washed and dry-cleaned materials (FAO, 2010). The costs of the selected items above are presented in Table 4.7. These costs were determined from details available from the farms.

4.2.3 Parameters and assumptions

The parameters and figures used in the calculations are available in Table 4.1 and the assumptions used are listed below:

Table 4.1: Farm targets for a 122-sow farrow-to-finish unit.

Production Parameters	Target	Farm used	Interference
Reproduction			
Number of productive sows	140	122	<95%
Farrowing interval	147 days	150 days	>190 days
Farrowing index	2.35	2.25	<2.0 and >2.4
Weaning to service interval	5 days	5 days	>16 and <26 days
Repeat mating (return to heat 18-24 days post-mating)	10%	12%	>15%
Empty days/sow/annum	34 days	36 days	<24 and >40 days
Abortion	<1%	2%	>5%
False pregnancy detected (after 70-80 days)	1%	2%	
Farrowing rate	>87%	96%	<75%
Sow parity at culling*	6-7	12	
Sow death per annum	2%	1%	>5%
Number of boars (service, sniff and replacement)	9	9	>11
Number of mating per week	7	7	<3 and >9
Replacement rate (sow)*	33% (1/3 per years)	25%	<25 and >50%
Replacement rate (boars)*	50% (1/2 per year)	20%	<30 and >60%
Number of replacement gilt per annum	33% (47)	32.8% (40)	<28% and >50%
Age at first service (gilts)	240	270	<240 and >300
Number of replacement boars per annum*	5	2	<3 and >7
Farrowing house performance			
Number of litter/sow/year	2.35	2.25	<2.0 and >2.4
Number of piglets/litter born alive (23/annum)	11	10.25	<8
Number of stillborn and mummies (1/litter)	7%	2%	>10%
Piglet mortality before weaning (2.31/sow/year)*	10%	22%	>15%
Total piglets weaned/sow/year (9.75/sow/litter)	23.27	≈22	<18
Age at weaning (piglets)	4 weeks	4 weeks	
Age at transfer to weaner house (piglets)	4 weeks	4 weeks	>6-8 weeks
Total numbers of piglets born alive/122 sows/year (2.35 × 0.96 × 122 × 11)	3,028	2,701	2,202
Total weaners/122 sows/year (2.25 × 0.96 × 122 × 9.75)	2,839	2,569	2,196
Feeding herd performance			
Percentage loss of weaners before sale as porkers	0- <1%	<0.05%	>3%
Percentage of porkers sold	>99%	≈100%	<97%
Porkers sold per sow per year	≈ 21	≈ 22	<18
Total number of porkers sold/year	2562	2569	
Feed			
Feed/sow/annum (including input for piglets)	1.20tons	1.25tons	>1.4tons
Feed per 122 sow-unit per year (1.2 ton/sow/year) including feed for boars	146.4tons	152.5tons	>170.8tons
Feed for 9 boars per annum	Included in sow and boar feed.		
Piglets (Creep feed @ 1kg per litter)	≈ 287kg	≈ 275kg	<244kg
Feed per porkers (weaner to porker at 1.4kg/day)	225kg	199.7kg	262.5kg
Feed for all porkers (weaner to porker)	57.65tons	51.30tons	
Mean annual cost per kilogramme of meat from porkers (at 71.5 - 75% meat from live weigh a 70kg pig will yield ≈50kg of pork)			US$2.47/kg
Mean annual cost per kilogramme of meat from culled sows (at 74 - 75% meat from live weight, a 250kg boar will yield ≈185kg of pork)			US$1.35/kg
Mean annual cost per kilogramme of meat from culled boar (at 75 - 77% meat from live weight, a 280kg pig will yield ≈210kg of pork)			US$0.45/kg

*Parameters with asterisk (*) indicate that the values from the used farm are either too high or too low. In such circumstances, standard values from the target were substituted for used farm values.

Production parameters were adapted using Carr, (1998); Stalder et al., (2010) or from personal communication with experts and field survey of selected farms.

The exchange rates of Nigerian Naira (₦.k) of ₦152.00 =US$1.00 was used for all calculations.

1. Each sow will produce an average of 10.25 piglets per litter and wean 9.75 piglets. A 2% case of stillborn/mummies will occur per sow on average in the farm (Table 4.1).
2. All cases of sudden death for the period of the assessment will be directly as a result of ASF or causes associated with it. ASF will ultimately lead to 100% mortality in infected farms either due to direct mortality caused by the disease or stamping-out policy implemented on the farm.
3. The sow will be replaced after the sixth parity and at an average age of ≈ 3 years. The boar will be replaced after 2 years of age.
4. Each porker will have a finishing liveweight of ≈ 70kg at 150 days. Culled sows will have a liveweight of ≈ 250kg while the culled boar will have a liveweight of ≈ 280kg. The prevailing price of prime cut for porkers of US$2.47, culled sow (US$1.35) and culled boars (US$0.45) will remain constant.
5. The lifespan of a standard fence erected will be 20 years. Depreciation is calculated using linear equation and mean valuation (1x + 2x + 3x + 4x +5x + 6x + 7x + 8x + 9x + 10x……….. 20x) whereas the total value of such materials will be divided by the lifespan of the materials and the mean value will be deducted in each year. The value of the fence will be zero after 20 years and that of the cemented tyre dip will be zero after the 10th year.
6. Depreciation in stock values was determined by deducting the final market value of each stock from the initial value and dividing the total loss of value over 6-monthly periods (Table 4.2).
7. Since it is known that the outbreak can occur at any period during the operational year, a mid-year value was obtained and used for all farm stock.
8. Implementation of biosecurity will be 100% effective against the risk of infection with ASF.

Profit was assessed by deducting all input values and costs from output values and incomes (Tables 4.5, 4.6 & 4.7). Year outputs per input as well as operation mean year total over a three-year period were also calculated. Details of the calculations are available in tables 4.5-4.7.

Assuming that African swine fever outbreak will cause massive mortality approaching 100%, or that the remaining stock will be depopulated following an outbreak, a potential loss associated with ASF was calculated and is presented in table 4.7. The bases for the calculations are available as footnotes to the tables.

From the above costs, values and outputs, the benefit cost analysis (BCA) of implementation of biosecurity against ASF was evaluated and presented.

4.2.4 Sensitivity analysis

Since the annual farm operation exists in a dynamic environment and changes in other variables may affect the annual profitability of farm operations and consequently that of the BCA, a sensitivity analysis was conducted to assess the effect of biosecurity implementation on overall profitability in the event that:

1. Cost of biosecurity increases by 100%,
2. Feed price increases by 30 up to 75%,
3. Management cost (input costs excluding feed) increases by between 25 and 75%, and
4. Total margins from outputs are reduced by 10 up to 25%.

4.3 Results

4.3.1 Profitability of the 122-sow farrow-to-finish farm and losses associated with potential ASF infection

The annual mean costs of operation was US$295,075.80 and feed costs accounted for 71.99% of the total variable costs while fixed costs represented 31.13% of the total costs (Table 4.3). Labour cost accounted for 86.30% of the total fixed cost in this study. A mean output of US$404,713.30 was generated and the porkers (weaner grown in the farm to finisher stage) accounted for 82.38% of this total output (Table 4.4). Annual profit gradually increased from US$107,923.70 to US$111,126.30 over a three year period with a mean annual profit of US$109,637.40 (Table 4.5).

Should ASF infect a non-biosecure farm in the first year of operation, the farm will lose a total of US$910,836.70. It should be noted that part of this amount does not represent real incomes since the whole sum is inclusive of potential incomes associated with the expected outputs from the farm in the second and third years of operation. If the infection occurs in the second year, there is a possibility of losing up to US$579,312.50 while an infection of the farm in the third year will result in the loss of approximately US$233,690.70 (Table 4.4). The losses will include the costs of wasted inputs (feeds, veterinary costs, drugs, vaccines, transportation, bills and utilities), clean-up costs, pay-off to staff, facility rental cost, and some maintenance costs in addition to real and potential outputs expected from the farm.

Table 4.2 Six-monthly depreciation rates (in percentage) in the value of pigs based on expert survey and prevailing prices.

	Initial	Mid-year 1	End of year 1	Mid-year 2	End of year 2	Mid-year 3	End of year 3
Sow (10.06% eve 6 months)	US$628.74	US$565.49	US$502.24	US$438.99	US$375.74	US$312.49	US$249.23
Boar (22.38% every 6 months)	US$898.20	US$697.23	US$498.95	US$297.98	US$97.01	**US$697.23**	**US$498.95**

Sows will experience a devaluation of 60.36% over a three year period equivalent to 10.06% every half year. Boars will experience a devaluation of 89.50% over a two year period equivalent to 22.38% per half year. Boar's value drops drastically because of boar taint that is expressed in meat from boar. It is assumed that new sets of stud boars will have completely replaced the old boars by the end of year two hence the reversion to year 1 rates for the third year (indicated in bold).

Table 4.3. Mid-year values of stock and costs of other inputs over a three-year farm operation period

	Year 1	Year 2	Year 3	Mean total-year cos	Comments
Stock					The estimates for year two and three were all calculated based on an annual inflation rate of 5%, with the exception of labour costs.
Purchase value of 122 sows in production mid year	US$68,989.78	US$53,556.78 + US$2,677.84 (5% inflation margin)	US$38,123.78 + US$1,906.19 (5% inflation margin)	US$165,254.37 dividec by 3 = **US$55,084.79**	Mid-year value of sow was estimated using 10.06% depreciation value/6 months of the cost of 1 gilt (US$628.74). After 3 years, the sow will have depreciated by a total of 60.36% (US$379.51) of the original cost to US$249.23. For the sow, the total mid-year mean cost will be (Year 1 + Year 2 + Year 3 costs) divided by three
Purchase value of 9 stud/sniff boars mid year	US$6,275.07	US$2,681.82 + US$134.09 (inflation margin)	US$6,275.07 + US$313.75 (inflation margin)	US$15,679.80 divided 3 = **US$5,226.60**	Mid-year value of boar was estimated using 22.38% depreciation value/6 month of the cost of 1 new stud boar (US$898.20). After 2 years, the boar will have depreciated by a total of 89.50% (US$801.19) of the original cost to US$97.01 largely due to boar taint. In the third year, a new set of boar will be valued. For the boar, the total mid-year mean cost will be (Year 1 + Year 2 + Year 3 costs) divided by three
Purchase of 40 replacement gilts	US$25,149.60	US$26,407.08	US$27,727.43	**US$26,428.04**	At US$628.74 per in-gilt.
Purchase of 5 replacement boars	US$4,491.00	US$4,715.55	US$4,951.33	**US$4,719.29**	At US$898.20 per stud boar.
Subtotal	**US$104,905.50**	**US$90,173.20**	**US$79,297.60**	**US$91,458.70**	
Variable costs (feed)					
Feed per 122 sow per year (1.25 ton/sow/year) including 9 boars	US$57,073.13	US$59,926.79	US$62,923.13	**US$59,974.35**	152.5 tons @ US$374.25/ton
Piglets (Creep feed @ 1kg per litter)	US$288.17	US$302.58	US$317.71	**US$302.82**	At US$1,047.90/ton. A total of 275kg is needed per year at the current level of productivity.
Feed for the porkers (28 to 150 days)	US$19,199.03	US$20,158.98	US$21,166.93	**US$20,174.98**	51.30tons at US$374.25/ton
Subtotal	**US$76,560.30**	**US$80,388.40**	**US$84,407.80**	**US$80,452.20**	
Variable costs (others)					
Veterinary services + Medicines and vaccination	US$11,676.65	US$12,260.48	US$12,773.50	**US$12,270.21**	Based on 6 veterinary consultations per annum at a cost of US$449.10 per visit. Drugs, medicaments and vaccination cost US$8,982.04.
Transport	US$8,982.04	US$9,431.14	US$9,902.70	**US$9,438.63**	
Utilities	US$17,964.07	US$18,862.27	US$19,805.38	**US$18,877.24**	
Other miscellaneous expenses	US$2,838.54	US$2,980.47	US$3,129.49	**US$2,982.83**	
Subtotal	**US$29,784.70**	**US$31,273.90**	**US$32,837.60**	**US$31,298.70**	
Fixed costs					
Labour	US$71,856.29	US$79,041.92	US$86,946.11	**US$79,281.44**	Labour cost were calculated based on an annual increase of 10%
Facility rentals at US$374.25/month	US$4,491.02	US$4,715.57	US$4,951.35	**US$4,719.31**	Fixed at US$374.25/month
Maintenance costs and Repairs	US$7,485.03	US$7,859.28	US$8,252.24	**US$7,865.52**	
Subtotal	**US$83,832.30**	**US$91,616.80**	**US$100,149.70**	**US$91,866.30**	
Gross total of all expenses and costs	**US$295,082.80**	**US$293,452.30**	**US$296,692.70**	**US$295,075.90**	

An annual inflation of 5% was factored into all calculations, unless stated otherwise.

Table 4.4. Mid-year values of stock and prices of other outputs over a three-year farm operation period

Farm outputs and prices	Year 1	Year 2	Year 3	Mean total-yea price	Comments
Price of total meat from porkers sold/year	US$317,279.	US$333,143.	US$349,800.	**US$333,407.57**	Mean annual cost per kilogramme of meat from porkers is US$2.47/kg (at 71.5% meat from live weight, a 70kg pig will yield 50kg of pork). A total of 2,569 will be produced per annum.
Price of total culled sows (40	US$9,990.00	US$10,489.5	US$11,013.9	**US$10,497.83**	Mean annual cost per kilogramme of meat from culled sows is US$1.35/kg (at 74% meat from live weight, a 250kg boar will yield 185kg of pork)
Price of total culled boar (5)	US$472.50	US$496.13	US$520.94	**US$496.52**	Mean annual cost per kilogramme of meat from culled boar is US$0.45/kg (at 75% meat from live weight, a 280kg culled boar will yield 210kg of pork) Price per kg of pork from culled boar
Purchase value of 122 sow in production mid year	US$68,989.7	US$53,556.7 + US$2,677.8 (5% inflation margin)	US$38,123.7 + US$1,906.1 (5% inflation margin)	US$165,254.37 divided by 3 = **US$55,084.79**	Mid-year value of sow was estimated using 10.06% depreciation value/6 months of the cost of 1 gilt (US$628.74). After 3 years, the sow will have depreciated by a total of 60.36% (US$379.51) of the original cost to US$249.23. For the sow, the total mid-year mean cost will be (Year 1 + Year 2 + Year 3 costs) divided by three
Purchase value of 9 stud/ sniff boars mid year	US$6,275.07	US$2,681.82 US$134.09 (inflation margin)	US$6,275.07 US$313.75 (inflation margin)	US$15,679.80 divided by 3 = **US$5,226.60**	Mid-year value of boar was estimated using 22.38% depreciation value/6 month of the cost of 1 new stud boar (US$898.20). After 2 years, the boar will have depreciated by a total of 89.50% (US$801.19) of the original cost to US$97.01 largely due to boar taint. In the third year, a new set of boar will be valued. For the boar, the total mid-year mean cost will be (Year 1 + Year 2 + Year 3 costs) divided by three
Total sales value per annur	**US$403,006.**	**US$403,179.**	**US$407,954.**	**US$404,713.30**	

All input and output costs and prices were based on the prevailing costs and prices. All costs and prices were translated in American Dollars and rounded off to the nearest Cent. The exchange rates of Nigerian Naira (₦.k) of ₦152.00 =US$1.00 was used for all calculations.

Table 4.5. Profit margins per annum over the three-year operation period

Parameter	Year 1	Year 2	Year 3	Mean year-total
Outputs	US$403,006.50	US$403,179.30	US$407,954.00	US$404,713.30
Inputs	US$295,082.80	US$293,452.20	US$296,692.70	US$295,075.90
Profit	**US$107,923.70**	**US$109,727.10**	**US$111,126.30**	**US$109,637.40**
Year Output/Inpu	1.366	1.374	1.375	1.372

Table 4.6. Potential losses associated with ASF outbreaks

Outbreak period	Potential losses	Output losses	Input losses	Total potential loss
Year 1	Lose ½ year 1 total outputs + some year 1 inputs + potential porkers from year 2 and year 3 without inputs of year 2 and 3	US$201,503.27 US$333,143.15 US$349,800.31	US$26,390.01	**US$910,836.70**
Year 2	Lose ½ year 2 total outputs + some year 2 inputs + potential porkers from year 3 outputs without inputs of year 3.	US$201,589.66 US$349,800.31	US$28,008.92	**US$579,312.50**
Year 3	Lose ½ year 3 outputs + some year 3 inputs	US$203,977.01	US$29,713.71	**US$233,690.70**

Mid-year values were used for all calculations. Inputs lost include the following: 1 month supply of feed (1/12); ¼ of cost of veterinary services and drugs as cost of breach of retainership contract; 1/6 of cost of transport as part of clean-up costs; ¼ of cost of utilities (in reduced bills and levies); 1 month cost of labour as pay-off to staff; total annual cost of renting of facility and 1 month maintenance costs (1/12).

Table 4.7. List and costs of items needed for biosecurity

Item	Unit cost	Total cost
Complete fencing of the piggery plus installation of doors and controlled access	US$22,455.09 for 20 years	US$1,122.75/annum.
Tyre dip for incoming vehicles	US$2,245.51 for 10 years	US$224.55/annum.
Cost of farm disinfectant per annum at the rate of 5L of disinfectant per month	US$35.95 per 5L	US$431.40/annum.
Cost of quarantine for incoming pigs per annum	Part of building cost/rented facility	
Provision of boots and clothes for 6 workers, 2 visitors, a manager and the farm director, total=10.	US$35.93 per overall and US$20.96 per pair of gumboot.	US$568.88/annum
Extra labour needed to ensure compliance	5% of normal labour cost	US$3,592.81/annum
Incentives to workers for compliance	2.5% normal labour cost	US$1,796.41/annum
Rat and other animals/insect control		US$500.00/annum
Correct disposal of farm mortalities and waste. Construction of a waste pit plus cover. It is assumed that a pit will be filled in three years.	Cost of pit = US$598.80	US$199.60/annum
Provision of hand and body washing facilities. 5L hand disinfectant every 2 months and 12 bars of antiseptic soap every month.	US$89.37 per 5L hand disinfectant and US$160 per 14 bars of antiseptic soap.	US$696.22/annum
Placement of restriction access notices	US$100.00	US$100.00
Secured feed store	Part of building cost	
Total		**US$9,232.62**

Based on a 5% annual inflation rate, the costs for biosecurity in the second and third year are US$9,694.25 and US$10,178.96 respectively. The potential cumulative costs of biosecurity for the 1st, 2nd, and 3rd year will be US$29,105.83, US$19,873.21 and US$10,178.96 respectively. The mean cost of Biosecurity over the three year period was US$9,701.94

4.3.2 Benefit of biosecurity against African swine fever compared with no-biosecurity using benefit-cost analysis (BCA)

The benefit cost ratio (BCR) of implementing biosecurity in a 122-sow farrow-to-finish unit can be determined in one of two ways:

BCR = {[Increase in net incomes + Decrease costs of operation] – [Decrease in net incomes + Increase cost of operation due to biosecurity]}. This formula is suitable for diseases that do not lead to 100% mortality of the herd.

Or

BCR = [Total losses per annum / total potential cost of biosecurity for that year], since it was assumed that no pig will be left after infection by ASF either due to ASF-associated mortality or stamping out policy.

Where the:

Increase in net incomes = profit retained, downtime cost saved, wasted feed and labour and other costs saved.

Decrease costs of operation = cost of disease management and clean-up costs.

Decrease in net incomes = extra costs of implementing biosecurity

Increase cost of operation due to biosecurity = assumed profit saved without biosecurity, value of total animal saved without biosecurity.

Since it was assumed that ASF will cause 100% mortality, the BCR of no-biosecurity will amount to zero because no animal will be saved for evaluation purposes. However, the BCR of biosecurity against an infection of ASF in the first year of operation will be US$910,836.70/US$29,105.80) = **31.29**

In the second year of operation, this value of biosecurity implementation against ASF is US$579,312.50/US$19,873.20 = **29.15**

In the third year of operation, this value will be US$233,690.70/US$10,179.00 = **22.96**

Over a three year operation, the BCR of biosecurity against ASF will be:

US$(910,836.70 + 579,312.50 + 233,690.70)/ US$(29,105.80 + 19,873.20 + 10,179.00) = US$1,723,839.90/US$59,158.00 = **29.14**

4.3.3 Sensitivity analysis

The inclusion of biosecurity in the farm operation will reduce the mean annual profit by 9.70%, however, this is justified in view of the potential benefit of 29 times expected over a three year period compared to not implementing biosecurity. If the cost of biosecurity increases by 100%, the mean annual profit will reduce by 19.42% while a 30% increase in cost of feed will reduce the mean annual profit by 33.86%. A 25% increase in cost of other variable and fixed costs will reduce the profit by 40.52% while a 75% increase in these costs will lead to loss of 102.14% (total loss of profit and an addition of 2.14%). If the incomes from the outputs are reduced by 10%, there will be a 50.21% loss in profit while a 25% loss in total outputs will lead to a loss of 110.95% of profit (Table 4.8).

Table 4.8. Sensitivity analyses of the implementation of biosecurity against ASF and price changes in a 122-sow farrow-to-finish piggery

Percentage change (Item)	Mean feed cc (US$)	Other variab (US$)	Fixed costs (US$)	Biosecurity cost (US$)	Purchase values of pigs (US$)	Mean total costs (US$)	Mean total outputs (US$	New annual profit (US$)	Initial profit without cost of biosecurity (US$)	Change in mean profit i US$	% reduction in profit
Current mean over three years	80,452	31,298	91,866	9,701	9,1458	304,777	404,71	99,935	109,637	9,701	9.70*
Cost of biosecurity increase by 100%	80,452	31,298	91,866	**19,403**	9,1458	314,479	404,71	90,233	109,637	19,403	19.42
Feed price increase by 30%	**104,587**	31,298	91,866	9,701	9,1458	328,913	404,71	75,799	109,637	33,837	33.86
Feed price increase by 50%	**120,678**	31,298	91,866	9,701	9,1458	345,003	404,71	59,709	109,637	49,928	49.96
Feed price increase by 75%	**140,791**	31,298	91,866	9,701	9,1458	365,116	404,71	39,596	109,637	70,041	70.09
25% increase in cost of management (other variables and fixed costs)	80,452	**39,123**	**114,832**	9,701	9,1458	335,569	404,71	69,144	109,637	40,493	40.52
50% increase in cost of management (other variables and fixed costs)	80,452	**46,948**	**137,799**	9,701	9,1458	366,360	404,71	38,353	109,637	71,284	71.33
75% increase in cost of management (other variables and fixed costs)	80,452	**54,772**	**160,765**	9,701	9,1458	397,151	404,71	7,561	109,637	102,075	102.14
Total margin from outputs is reduced by 10%	80,452	31,298	91,866	9,701	9,1458	304,777	**364,241**	59,464	109,637	50,173	50.21
Total margin from outputs is reduced by 15%	80,452	31,298	91,866	9,701	9,1458	304,777	**344,006**	39,228	109,637	70,408	70.45
Total margin from outputs is reduced by 20%	80,452	31,298	91,866	9,701	9,1458	304,777	**323,770**	18,992	109,637	90,644	90.70
Total margin from outputs is reduced by 25%	80,452	31,298	91,866	9,701	9,1458	304,777	**303,534**	-1,242	109,637	110,880	110.95

*Mean values of all costs and outputs over the three-year period were used for the sensitivity analysis viz. the cumulative addition of costs from year 1, 2 and 3 divided by three. *Changes in the mean profit for the current situation was due to the integration of costs of biosecurity which was not included in the initial calculations without biosecurity (see Tables 4.5,4. 7 and 4.9) .*

4.4 Discussion

In this study, we have demonstrated the profitability of a 122-sow pig farm and described an economic approach to preventing ASF virus infection at farm level. We are aware that farm profitability may not always be based on optimum productivity as the interplay of various factors may affect farm operations and lower maximum profitability; this effect is reduced in this analysis by the use of real farm data as the template to simulate profitability. An attempt to examine the cost effectiveness of intervention using biosecurity and a situation of no intervention for a probable African swine fever virus infection of the 122-sow unit indicated that intervention at farm level using biosecurity to prevent the introduction of ASF was far more effective than taking no action (benefit-cost ratio: 29.14).

Since ASF is currently endemic in Nigeria and in most of the West African states, and no vaccine is available to control the disease, it will be important to focus on preventing ASF infection using biosecurity. It should however be borne in mind that prevention of outbreaks of a disease like ASF is an interplay of diverse factors, including the effectiveness of biosecurity. Despite the fact that the facilities and tools needed for the implementation of farm-level biosecurity will come at a cost, the investment is justified in view of the outcomes that are derived from implementation of these measures. Furthermore, some of these facilities including fencing and tyre dips will be useful for a long time and for other purposes other than biosecurity. While the study focused on the benefit of biosecurity in preventing ASF infection alone, the biosecurity implementation will also prevent other infectious diseases like foot-and-mouth disease (FMD), classical swine fever (CSF), Aujeszky’s disease, swine vesicular disease (SVD), porcine circoviruses (PCV) and porcine reproductive and respiratory syndrome virus (PRRS).

Thus, the total overall benefits of biosecurity are likely to far outweigh the cost benefit analysis done in this study.

Babalobi and colleagues (2007) had previously described a combined mortality of over 91% in 306 pig farms in southwest Nigeria, and field observation has confirmed similar figures. A situation of this magnitude will come with loss of trade, redundancy of facility, psychosocial stress on the farmer and the potential to infect neighbouring farms. The cost of destruction of the remaining pigs and burying, as well as the disinfection of the farm following outbreaks of ASF will also add to the burden of ASF virus infection. The information provided by the result of this work will guide sound decision-making related to the allocation of funds to biosecurity implementation, in the face of other competing interests.

The benefit-cost analysis of biosecurity indicates that it is justified on economic grounds. In the smallholder farms survey earlier mentioned, we confirmed that there have been several/repeated outbreaks of ASF and currently, there are a combined total of less than 100,000 pigs in the survey area, the ASF status of which is unknown to the farmers. However, the recent findings of Fasina *et al*. (2010) revealed that the prevalence of ASF on the farms is very high (50% seropositivity and 97% positive for ASF virus genome). Although we used a value of 100% mortality to calculate our values in this analysis, other workers have reported similar or lesser percentages of mortality in severe cases (Dixon *et al*., 1994; Penrith *et al*., 2004; Bastos *et al*., 2004; Babalobi *et al*., 2007), and we are aware that less acute forms of ASF exist, a situation that may perpetuate itself in pig farms and cause reduced but continuous economic losses (Penrith *et al*., 2004). It is our opinion that biosecurity at farm level will be better than no intervention irrespective of the form of ASF virus infecting a farm.

The sensitivity analysis of this model has proved that even with the inclusion of biosecurity in the farm operation, the proposed project will survive the additional variations that may cause foreclosure. A 100% increase in cost of biosecurity will cause a 19.42% reduction in profit (US$19,403.90) and this may save a potential value of US$910,836.70 in stock and farm operations. However a greater than 50% increase in cost of feed as well as an output margin reduced by 15% or more will have a negative effect on the implementation of biosecurity at farm level. It is unlikely that this margin of increase on feed cost will happen without government intervention in view of the similar food resources required by humans and pigs.

Although the focus of this study is on economic analysis alone, other forms of losses which can not be quantified in economic terms exist. The psychosocial stress, loss of health and human (pig farmers) death following the complete loss of livelihood is difficult to quantify. If it were possible to quantify the above impacts economically, we believe that the benefit-cost of biosecurity would rise significantly.

This model of biosecurity herein reported can be favourably implemented at smallholder farm-level since it is easily adaptable, less costly and socially acceptable (Ekue and Wilkinson, 1990). This model if combined with good management practices will be of tremendous benefit to the farmers. It should be possible to train extension agents, veterinarians and government agricultural workers to communicate the message of biosecurity, including its financial benefit to pig farmers. The use of community leaders and cooperative unions may also assist in this regard. Finally, a case of ASF on a farm, if left uncontrolled will result in a huge loss of investment on a national scale since inter-farm and inter-regional spread is inevitable (Mannelli *et al*., 1997).

Acknowledgements

We wish to thank the farmers that cooperated by supplying information used in this study especially the Cooperative Unions at the Oke-Aro Pig Estate, Lagos, Nigeria and Mrs. G. Rossato and her team in Kelly Farms, Skeerpoort, South Africa.

4.5 References

Babalobi, O.O., B.O. Olugasa, D.O. Oluwayelo, I.F. Ijagbone, G.O. Ayoade, S.A. Agbede, 2007: Analysis and evaluation of mortality losses of the 2001 African swine fever outbreak, Ibadan, Nigeria. *Trop. Anim. Hlth Prod* 39, 533-542.

Bastos, A.D.S., M.-L. Penrith, F. Macome, F. Pinto, G.R. Thomson, 2004: Co-circulation of two genetically distinct viruses in an outbreak of African swine fever in Mozambique: no evidence for individual co-infection. *Vet. Microbiol.* 103, 169–182.

Carr, J., 1998: Farms targets per 100 sows. In: *Garth Pig Stockmanship Standards.* 5M Enterprises Ltd., Sheffield, UK.

Dixon, L. K., S. R. F. Twigg, S. A. Baylis, S. Vydelingum, C. Bristow, J. M. Hammond, G. L. Smith, 1994: Nucleotide sequence of a 55 kbp region from the right end of the genome of a pathogenic African swine fever virus isolate (Malawi LIL20/1). *J Gen Virol.* 75:1655-1684.

Ekue, N. F., P. J. Wilkinson, 1990: Absence of *Ornithodoros moubata*, the vector of African swine fever virus, from the main pig producing area of Cameroon. *Trop Anim Health Prod.* 22(2):127-31.

El-Hicheri K., 1998: Emergency Assistance on control and eradication of an outbreak of African swine fever in Western Nigeria. *Report of the FAO Consultancy Mission to Nigeria.* TCP/NIR/7822(E). FAO, Rome, December, 1998.

Fasina, F. O., D. Shamaki, A. A. Makinde, L. H. Lombin, D. D. Lazarus, S. A. Rufai, S. S. Adamu, D. Agom, V. Pelayo, A. Soler, A. Simón, A. J. Adedeji, M. B. Yakubu, S. Mantip, A. J. Benshak, I. Okeke, P. Anagor, D. C. Mandeng, B. O. Akanbi, A. A. Ajibade, I. Faramade, M. M. Kazeem, L. U. Enurah, R. Bishop, R. Anchuelo, J. H. Martin, C. Gallardo, 2010: Surveillance for African Swine Fever in Nigeria, 2006-2009. *Transboundary Emerging Dis,* 57(4): 244-253.

Food and Agricultural Organization of the United Nations, 2001: *The State of Food and Agriculture 2001*. FAO, Rome, Italy. Also available online at http://www.fao.org/docrep/003/x9800e/x9800e00.htm. Accessed on August 24, 2010.

Food and Agricultural Organization of the United Nations /World Organization for Animal Health (FAO/OIE), 2010: *Good Practices for Biosecurity in the Pig Sector: Issues and Options in Developing and Transition Countries*. FAO Animal Production and Health paper 169. FAO, Rome, 2010. Also available online at www.fao.org/docrep/012/i1435e/i1435e00.pdf. Accessed on August 24, 2010.

Jori, F., Bastos, A.D.S. 2009: Role of wild suids in the epidemiology of African swine fever. *EcoHealth*., 6:296-310.

Marsh W., 1999: The economics of animal health in farmed livestock at the herd level. *Rev. sci tech Off int Epiz.,* 18(2):357-366.

Mannelli, A., S. Sotgia, C. Patta, A. Sarria, P. Madrau, L. Sanna, A. Firinu, A. Laddomada, 1997: Effect of husbandry methods on seropositivity to African swine fever virus in Sardinian swine herds. *Prev vet Med*. 32:235-241

Olugasa, B. O., I. F. Ijagbone, 2007: Pattern of spread of African swine fever in south-western Nigeria. *Veterinaria Italiana* 43(3):621-628

Penrith, M-L., G. R. Thomson, A. D. S. Bastos, 2004: African swine fever. In: Coetzer, J. A. W., Tustin, R. C. (Eds.), *Infectious Diseases of Livestock with Special Reference to Southern Africa*, 2nd ed. Oxford University Press, Cape Town, pp 1087–1119.

Stalder, K., L. Engblom, J. Mabry, 2010: Benchmarking sow lifetime productivity. Available at http://www.benchmark.farms.com/Benchmarking_Sow_Lifetime_Productivity.html . Accessed on August 22, 2010.

Tambi, E. N., O. W. Maina, A. W. Mukhebi, T. F. Randolph, 1999: Economic impact assessment of rinderpest control in Africa. *Rev. sci tech Off int Epiz.,* 18(2):458-477.

Tisdell, C. A., S. R. Harrison, G. C. Ramsay, 1999: The economic impacts of endemic diseases and disease control programmes. *Rev. sci tech Off int Epiz*. 18(2):380-398.

Vial, L., B. Wieland, F. Jori, E. Etter, L. Dixon, F. Roger, 2007: African swine fever virus DNA in soft ticks, Senegal. *Emerg Infect Dis*. 13(12):1928-1931.

5.0 RISK FACTORS FOR FARM-LEVEL AFRICAN SWINE FEVER INFECTION IN MAJOR PIG-PRODUCING AREAS IN NIGERIA, 1997-2011

Fasina, F.O[1, 2*]., Agbaje, M[3]., Ajani, F.L.O[3]., Talabi, O.A[3]., Lazarus, D.D[1, 4]., Gallardo, C[5]., Thompson, P.N[1]., Bastos, A.D.S[2].

[1] Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa.

[2] Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, South Africa.

[3] College of Veterinary Medicine, University of Agriculture, Abeokuta, Ogun State, Nigeria.

[4] African Swine Fever Project Implementation Task Team, National Veterinary Research Institute, Vom, Plateau State, Nigeria.

[5] Community Reference Laboratory for African swine fever, CISA-INIA, Valdeolmos, 28130, Madrid, Spain.

*Corresponding Author: Dr Fasina, F. O., Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Tel: +27 12 529 8069; Email: daydupe2003@yahoo.co.uk

Aim: *To identify the risk factors for African swine fever in Nigeria, and determine the high risk pig areas and suggest necessary control strategies.*

This paper has been published in ***Preventive Veterinary Medicine*** with the document optical identifier number: 10.1016/j.prevetmed.2012.05.011

Preventive Veterinary Medicine 107 (2012) 65–75

Contents lists available at SciVerse ScienceDirect

Preventive Veterinary Medicine

journal homepage: www.elsevier.com/locate/prevetmed

Risk factors for farm-level African swine fever infection in major pig-producing areas in Nigeria, 1997–2011

F.O. Fasina[a,b,*], M. Agbaje[c], F.L. Ajani[c], O.A. Talabi[c], D.D. Lazarus[a,d], C. Gallardo[e], P.N. Thompson[a], A.D.S. Bastos[b]

[a] Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa
[b] Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, South Africa
[c] College of Veterinary Medicine, University of Agriculture, Abeokuta, Ogun State, Nigeria
[d] African Swine Fever Project Implementation Task Team, National Veterinary Research Institute, Vom, Plateau State, Nigeria
[e] Community Reference Laboratory for African Swine Fever, CISA-INIA, Valdeolmos, 28130 Madrid, Spain

ARTICLE INFO

Article history:
Received 23 September 2011
Received in revised form 22 May 2012
Accepted 23 May 2012

Keywords:
African swine fever
Farm-level infection
Case–control
Pig
Nigeria

ABSTRACT

African swine fever (ASF) is an economically devastating disease for the pig industry, especially in Africa. Identifying what supports infection on pig farms in this region remains the key component in developing a risk-based approach to understanding the epidemiology of ASF and controlling the disease. Nigeria was used for this matched case–control study, because there is perpetual infection in some areas, while contiguous areas are intermittently infected. Risk factors and biosecurity practices in pig farms were evaluated in association with ASF infection. Subsets of farms located in high-density pig population areas and high-risk areas for ASF infection were randomly selected for analysis. Most plausible risk factor variables from the univariable analysis included in the multivariable analysis include: owner of farm had regular contact with infected farms and other farmers, untested pigs were routinely purchased into the farm in the course of outbreaks, there was an infected neighbourhood, other livestock were kept alongside pigs, there was a presence of an abattoir/slaughter slab in pig communities, wild birds had free access to pig pens, tools and implements were routinely shared by pig farmers, there was free access to feed stores by rats, and feed was purchased from a commercial source.

Only the presence of an abattoir in a pig farming community (OR = 8.20; $CI_{95\%}$ = 2.73, 24.63; P < 0.001) and the presence of an infected pig farm in the neighbourhood (OR = 3.26; $CI_{95\%}$ = 1.20, 8.83; P = 0.02) were significant. There was a marginally significant negative association (protective) between risk of ASF infection and sharing farm tools and equipment (OR = 0.35; $CI_{95\%}$ = 0.12, 1.01; P = 0.05).

Of the 28 biosecurity measures evaluated, food and water control (OR = 0.14; $CI_{95\%}$ = 0.04, 0.46; P < 0.001), separation/isolation of sick pigs (OR = 0.14; $CI_{95\%}$ = 0.04, 0.53; P = 0.004) and washing and disinfection of farm equipment and tools (OR = 0.27; $CI_{95\%}$ = 0.10, 0.78; P = 0.02) were negatively associated (protective) with ASF infection. Consultation and visits by veterinarian/paraveterinarians when animals were sick (OR = 8.11; $CI_{95\%}$ = 2.13, 30.90; P = 0.002), and pest and rodent control were positively associated with ASF infection of Nigerian farms (OR = 4.94; $CI_{95\%}$ = 1.84, 13.29; P = 0.002).

* Corresponding author at: Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa. Tel.: +27 12 529 8069; fax: +27 12 529 8396.
E-mail address: daydupe2003@yahoo.co.uk (F.O. Fasina).

0167-5877/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.prevetmed.2012.05.011

5.0.1 ABSTRACT

African swine fever (ASF) is an economically devastating disease for the pig industry, especially in Africa. Identifying what supports infection on pig farms in this region remains the key component in developing a risk-based approach to understanding the epidemiology of ASF and controlling the disease. Nigeria was used for this matched case-control study, because there is perpetual infection in some areas, while contiguous areas are intermittently infected. Risk factors and biosecurity practices in pig farms were evaluated in association with ASF infection. Subsets of farms located in high-density pig population areas and high-risk areas for ASF infection were randomly selected for analysis. Most plausible risk factor variables from the univariable analysis included in the multivariable analysis include: owner of farm had regular contact with infected farms and other farmers, untested pigs were routinely purchased into the farm in the course of outbreaks, there was an infected neighbourhood, other livestock were kept alongside pigs, there was a presence of an abattoir/slaughter slab in pig communities, wild birds had free access to pig pens, tools and implements were routinely shared by pig farmers, there was free access to feed stores by rats, and feed were purchased from a commercial source

Only the presence of an abattoir in a pig farming community (OR = 8.20; $CI_{95\%}$ = 2.73; 24.63; P < 0.001) and the presence of an infected pig farm in the neighbourhood (OR = 3.26; $CI_{95\%}$ = 1.20; 8.83; P = 0.02) were significant. There was a marginally significant negative association (protective) between risk of ASF infection and sharing farm tools and equipment (OR = 0.35; $CI_{95\%}$ = 0.12; 1.01; P = 0.05).

Of the 28 biosecurity measures evaluated, food and water control (OR = 0.14; $CI_{95\%}$ = 0.04, 0.46; P < 0.001), separation/isolation of sick pigs (OR = 0.14; $CI_{95\%}$ = 0.04, 0.53; P

= 0.004) and washing and disinfection of farm equipment and tools (OR = 0.27; $CI_{95\%}$ = 0.10, 0.78; P = 0.02) were negatively associated (protective) with ASF infection. Consultation and visits by veterinarian/paraveterinarians when animals were sick (OR = 8.11; $CI_{95\%}$ = 2.13, 30.90; P = 0.002), and pest and rodent control were positively associated with ASF infection of Nigerian farms (OR = 4.94; $CI_{95\%}$ = 1.84, 13.29; P = 0.002).

The presentation of sick and unthrifty pigs for slaughter at abattoirs, farmers' inadvertent role, an infected neighbourhood, a pig to pig contact, rodents and wild birds may contribute to infections of farms, whereas washing, disinfection of tools, food and water control, and separation of sick pigs reduces the likelihood of infections. Underlying reasons for these observations and strategies for control are discussed.

Keywords: African swine fever, farm-level infection, case-control, pig, Nigeria.

5.1 Introduction

The African swine fever virus (ASFV), an *Asfivirus* of the *Asfarviridae* family, continues to spread across Nigerian farms, causing sporadic outbreaks of African swine fever (ASF), with associated mortalities. Historically, the virus first made an apparently unsustained incursion into Nigeria in 1973, wiping out the infected pig herd and then becoming extinct (Owolodun et al., 2010). However, there was a resurgence of outbreaks in West Africa from 1996 onwards, with the virus entering Nigeria in August/September 1997. ASF has remained a problem in Nigerian piggeries since then (see Figures 5.1a & b). Persistent infections with ASFV appear to recur in the core pig-producing areas of the country. A theory of geographical contiguity (in other words, infection in one state is highly likely to cause an outbreak in the neighbouring state(s)) has been proposed and is supported by the incidence of infections (see Figures 5.1a & b).

Nigeria, like many West and Central African countries where ASF is endemic, experiences intermittent infections. Very recently, in 2011, the Republics of Chad, Kenya, Cameroun, Tanzania and Malawi were infected, with huge fatalities in pigs and consequently significant loss of income and employment opportunities (OIE, 2011). Indeed, such infections have an overall effect on the pig industry worldwide, because they limit opportunities to explore external markets and because of the potential spread of the disease and the increasing rapidity/possibilities of inter-continental contamination. Some of the previously ASF-free areas of the world, including parts of Russia and the Caucasian regions, are now experiencing repeated infections of ASF (Rowlands et al., 2008; OIE, 2011).

Intensive efforts have been made in the use of genetic epidemiology to analyse the ASF viruses circulating in different parts of Africa in order to gain an significant understanding of the relation between and geographic spread of each circulating genotype (Bastos et al., 2003; Bastos et al., 2004; Lubisi et al., 2005; Boshoff et al., 2007; Lubisi et al., 2007; Gallardo et al., 2009; Owolodun et al., 2010; Gallardo et al., 2011). However, the causes/factors that support the continued circulation of ASF viruses in pig herds in various parts of Africa in general and in Nigerian pig populations in particular remain poorly understood or at best hypothetical.

Only three types of epidemiological cycles have been described for ASFV to date:

(i) an ancient sylvatic cycle that primarily involves warthogs (*Phacochoerus africanus*) and argasid ticks of the genus *Ornithodoros*, with occasional spill-over to domestic pigs;

(ii) a cycle in domestic pigs that involves *Ornithodoros* ticks inhabiting pig sties; and

(iii) a cycle in domestic pigs which occurs without the involvement of sylvatic hosts or vectors (Penrith et al., 2004).

ASFV has previously been detected as a spill-over infection via *Ornithodoros sonrai* ticks in Senegal (Vial et al., 2007) and from wild suids in Nigeria (Luther et al., 2007a), but the first two cycles have not been widely linked to the epidemiology of ASF in West Africa. Thus, a greater understanding of the factors responsible for the continued presence and maintenance of the virus in domestic pig populations (without the agency of sylvatic hosts and tick vectors) is vital for achieving regional control and eradication.

In order to investigate the risk factors for ASF in Nigerian pig herds and to identify high-risk farms, we carried out a case-control study that focused on environmental risks and biosecurity in pig herds under various farming conditions in the hope that the results of

this analysis will inform the formulation of policies to support ASF control efforts and reduce the burden of ASF in West Africa.

5.2 Materials and methods

5.2.1 Study locations and mapping of the spread of ASF, 1997-2009

Samples from suspected ASF outbreaks submitted to the National Veterinary Research Institute (NVRI) in Vom, Nigeria, as well as samples obtained through two active surveillance programmes in which both suspect and apparently healthy pigs were evaluated between 2006 and 2009, form the basis of this study. A total of 1,279 sera and 1,332 pooled tissues (767 tissues from the 2006-7 active surveillance; 269 tissues from the 2002-7 passive surveillance; and 296 tissues from the 2006-9 active surveillance) were collected from 19 states (see Figures 5.1a & b) and were analysed at the NVRI diagnostic laboratories. Duplicate samples of selected tissues and all sera were dispatched to the Centro de Investigación en Sanidad Animal (CISA-INIA) in Valdeolmos, Madrid, Spain, for quality control and duplicate confirmation of positive and negative samples. These results were supplemented by data from peer-reviewed literature and commissioned reports on ASF outbreaks in Nigeria between 1997 and 2009 (El-Hicheri, 1998; Luther et al., 2007b; Owolodun et al., 2007; Owolodun et al., 2010; Fasina et al., 2010). The data were first filtered to exclude duplications and were then combined for the purposes of the spatio-temporal mapping of suspected and confirmed outbreaks (see Figures 5.1a & b).

From the list of high-risk locations investigated, four states were selected for inclusion in a matched case-control study. These included Imo (in south-east Nigeria), Kebbi (in

north-west Nigeria), Lagos (in south-west Nigeria) and Taraba (in north-east Nigeria). The chosen states are representative of the distributions of outbreak locations and pig populations in Nigeria, and they were chosen as subsets of high-risk locations and high density pig areas. Within the selected states, case and control farms were subsequently selected as indicated below.

5.2.2 Case farm definition

Cases were defined in accordance with the international regulations for confirming ASF (OIE, 2008). Briefly, a farm is considered a potential case farm if it meets the following criteria:

(i) clinical signs consistent with ASF infection – high fever, depression, loss of appetite, heightened abortion, sudden death and loss of body condition;

(ii) pathological signs – extensive haemorrhage of the visceral organs, including the lymph nodes, spleen and kidneys; and

(iii) one or more animals from the farm being diagnosed positive for the presence of the ASF viral genome by polymerase chain reaction (PCR), in combination with at least one of three diagnostic tests: indirect ELISA, immunoblotting assay, immunofluorescence assay and virus isolation.

Some of the case farms reported repeated outbreaks of ASF between 1997 and 2010. Detailed results of the tests have been reported by Fasina et al. (2010). All the case farms included in the study were selected randomly from among the farms confirmed positive for the presence of the ASF genome, antibodies or virus between 2008 and 2011 (n = 343). They originated from four states (Imo, Kebbi, Lagos or Taraba). A total of 120

questionnaires were sent out to collect data from case farms, but only 72 farms finally qualified for inclusion as case farms. Reasons for the elimination of the responses from the remaining 48 farms included inconsistent reports and double entries and/or incomplete entries on the questionnaires. A further three case farms were eliminated because no matched control farms (see below) were available for them, leaving 69 case farms for the analysis.

5.2.3 Control farm description

Control farms were matched with case farms on the basis of farm location (Imo, Kebbi, Lagos or Taraba) and farm population size (<50 pigs; 51-100 pigs or >100 pigs). Eligible controls were farms which were within the infected or surveillance zones of ASF-infected farms and which were at the risk of infection due to close proximity (within 500 m and up to a 5 km perimeter) to an infected farm. These farms had similar population characteristics to the case farms (see Table 5.1) and were clustered geographically, like the case farms. Samples from these farms were collected at the same time as those from the case farms, but the samples tested negative for the ASF genome, antibodies or virus, using a combination of the clinico-pathological and laboratory diagnostic tests mentioned above.

A control farm was a pig farm under the management of one farmer with one or more pigs managed together as a group, where animals were at risk of infection with ASF, but consistently tested negative to ASF both serologically and virologically for the duration of the study period. Additional qualifications for eligibility included the presence of pigs on the farm between 2008 and 2011, when the case farms were sampled, and

confirmation of the independent management of the control and case farms. Of the 120 questionnaires sent out, 86 were returned, but missing data rendered 26 unusable, leaving 60 control farms for the analysis.

5.2.4 Data collection: the questionnaire

Epidemiological data were gathered by means of a self-rated closed-ended questionnaire completed by farmers. Prior to the administration of the closed-ended questionnaire, farmers met in groups and the purpose of the questionnaire was discussed. Each farmer was then asked to fill in the questionnaire, without interference, at his/her individual farm to avoid personal and diplomatic biases. Matched variables (based on farm size and location) were collected and grouped in categories:

(i) farm characteristics;

(ii) farm operations; and

(iii) self-reported biosecurity measures (see Appendix C).

5.2.5 Statistical analysis

Each potential risk factor and biosecurity measure was coded as a dichotomous independent variable. The odds of being an ASF case based on serology and virology was then modelled as a function of the dichotomous risk factors and biosecurity measures, using conditional logistic regression models, as suggested by Hosmer and Lemeshow (1989). The initial screening of potential risk factors for ASF infection and biosecurity measures to prevent infection was performed using univariable conditional logistic regression.

Variables associated with the outcome (ASF virus infection) at $P \leq 0.2$ were considered for inclusion in the multivariable conditional logistic regression models. Independent variables were tested for pairwise associations, using a two-tailed chi-square test. Two multivariable conditional logistic regression models were developed: one for the risk factors and one for the biosecurity measures. A backward selection procedure was applied using a selection threshold of $P \leq 0.05$ to reduce the number of variables in the model. All the excluded variables were then individually re-tested in the model and retained if they were significant.

Farm population size was then entered into each model as a continuous variable to test for residual confounding effects and was retained if it resulted in more than a 10% change to the coefficient for any of the other remaining predictors. Interactions between farm size and each of the remaining predictors were also tested and retained in the model if they were significant.

The fit of the final models was assessed using the Akaike information criterion (AIC) and the Bayesian information criterion (BIC), since the Hosmer-Lemeshow goodness-of-fit test is inappropriate for conditional logistic regression models, and the *m*:*n* matching precluded the use of leverage and influence statistics. In the final models, the odds ratios (OR), *P*-values and 95% confidence interval were reported. All statistical analyses were done using Stata 11 (StataCorp, College Station, Texas, USA).

5.3 Results

5.3.1 Spatial and temporal patterns

The mapping of laboratory-confirmed cases of ASF revealed that some locations were perpetually infected, while states contiguous to those locations were intermittently infected (see Figures 5.1a & b). The localities defined as "perpetually infected" for the period of study coincided roughly with high-density pig producing and marketing areas.

5.3.2 Case-control study

The matching pattern in the final dataset used for analysis was *m*:*n*, in other words, one or more case farms were matched with one or more control farms. There were 11 matched groups, with between 1 and 14 case farms and between 2 and 10 control farms per group. The population characteristics of the case and control farms are shown in Table 5.1.

The results of the univariable analysis of risk factors are set out in Table 5.2. The following variables were selected for inclusion in the multivariable model: the owner of the selected farm has regular contact with infected farms and other farmers on such farms, routine purchase of mostly untested pigs which are brought to the farm in the course of outbreaks, an infected neighbourhood, the keeping of other livestock alongside pigs, the presence of an abattoir/slaughter slab in pig communities, wild birds having free access to pig pens, tools and implements routinely being shared by pig farmers, free access to feed stores by rats, and the purchasing of feed from a commercial source (Table 5.2).

The final conditional logistic regression model for the risk factors is shown in Table 5.3.

The presence of an abattoir in the pig farm area was strongly associated with increased odds of ASF infection (OR = 8.20; $CI_{95\%}$ = 2.73; 24.63; $P < 0.001$). In addition, pig farms were at higher risk of infection if there was an infected pig farm present in the neighbourhood (OR = 3.26; $CI_{95\%}$ = 1.20; 8.83; $P = 0.02$). However, there was a marginally significant negative (protective) association between the risk of ASF infection in pig communities and the sharing of farm tools and equipment (OR = 0.35; $CI_{95\%}$ = 0.12; 1.01; $P = 0.05$).

For the self-reported biosecurity measures, based on the univariable analysis, several factors were selected for inclusion in the multivariable model (see Tables 5.4 and 5.5). The final conditional multivariable analysis (see Table 5.5) shows that only food and water control (OR = 0.14; $CI_{95\%}$ = 0.04, 0.46; P < 0.001), separation or isolation of sick pigs (OR = 0.14; $CI_{95\%}$ = 0.04, 0.53; P = 0.004) and washing and disinfection of equipment and tools (OR = 0.27; $CI_{95\%}$ = 0.10, 0.78; P = 0.02) showed negative (protective) associations with ASF infection. Consultation with and visits by veterinarians or paraveterinarians when animals were sick (OR = 8.11; $CI_{95\%}$ = 2.13, 30.90; P = 0.002), as well as pest and rodent control measures (OR = 4.94; $CI_{95\%}$ = 1.84, 13.29; P = 0.002), were positively associated with ASF infection of farms.

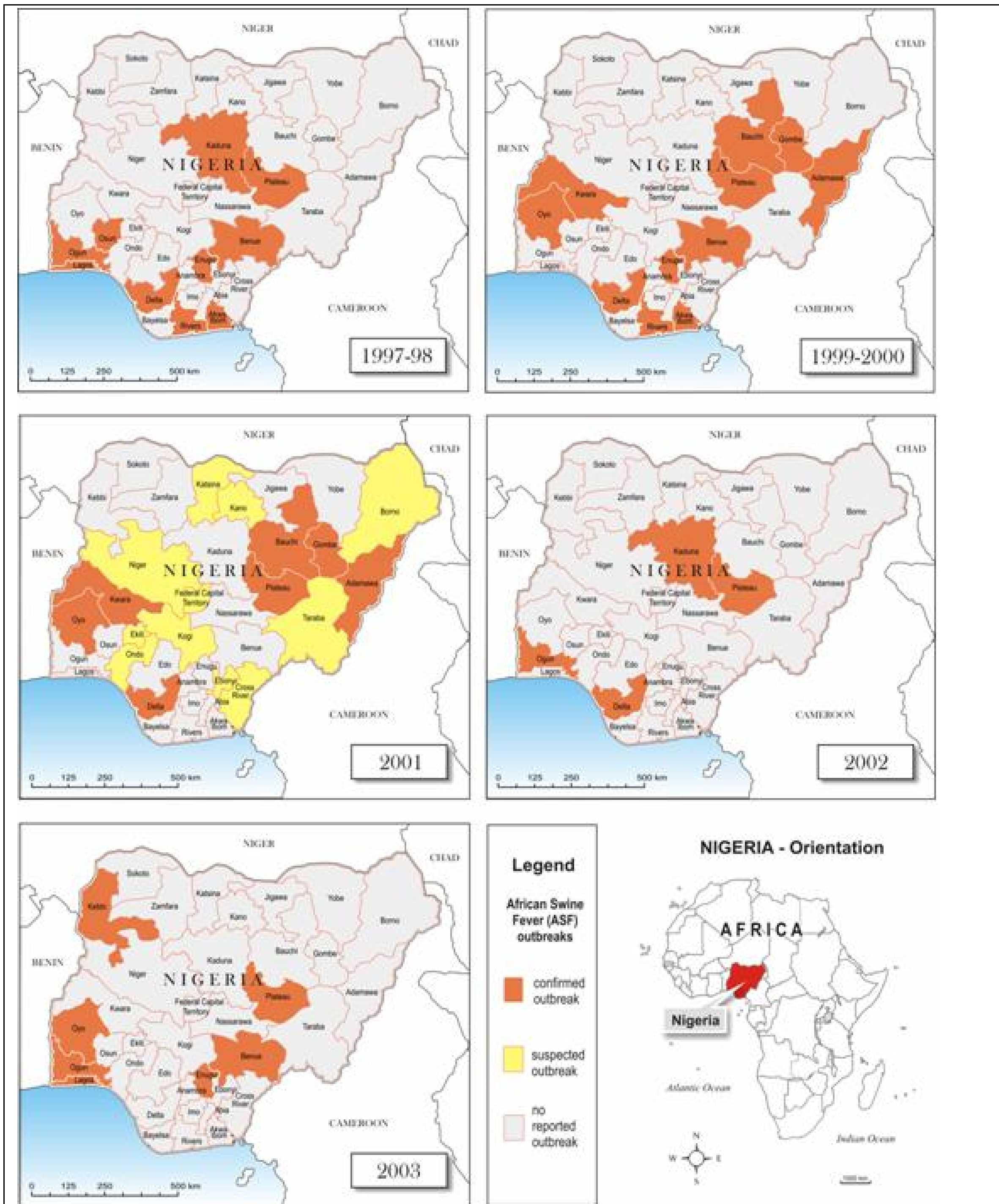


Figure 5.1a. Spatio-temporal representation of reported and confirmed outbreaks of ASF outbreaks in Nigerian states, 1997-2003.

The index outbreak occurred in the Ogun/Lagos axis in September 1997 and spread to other locations, eventually covering 9 states. Since these initial outbreaks, ASF has been reported (yellow) and confirmed (red) annually in Nigeria. It should be noted that several farmers may slaughter their sick pigs without reporting the outbreak. In that case, the true spatial prevalence of yearly intermittent and sporadic outbreaks may cover larger areas than those represented on the map.

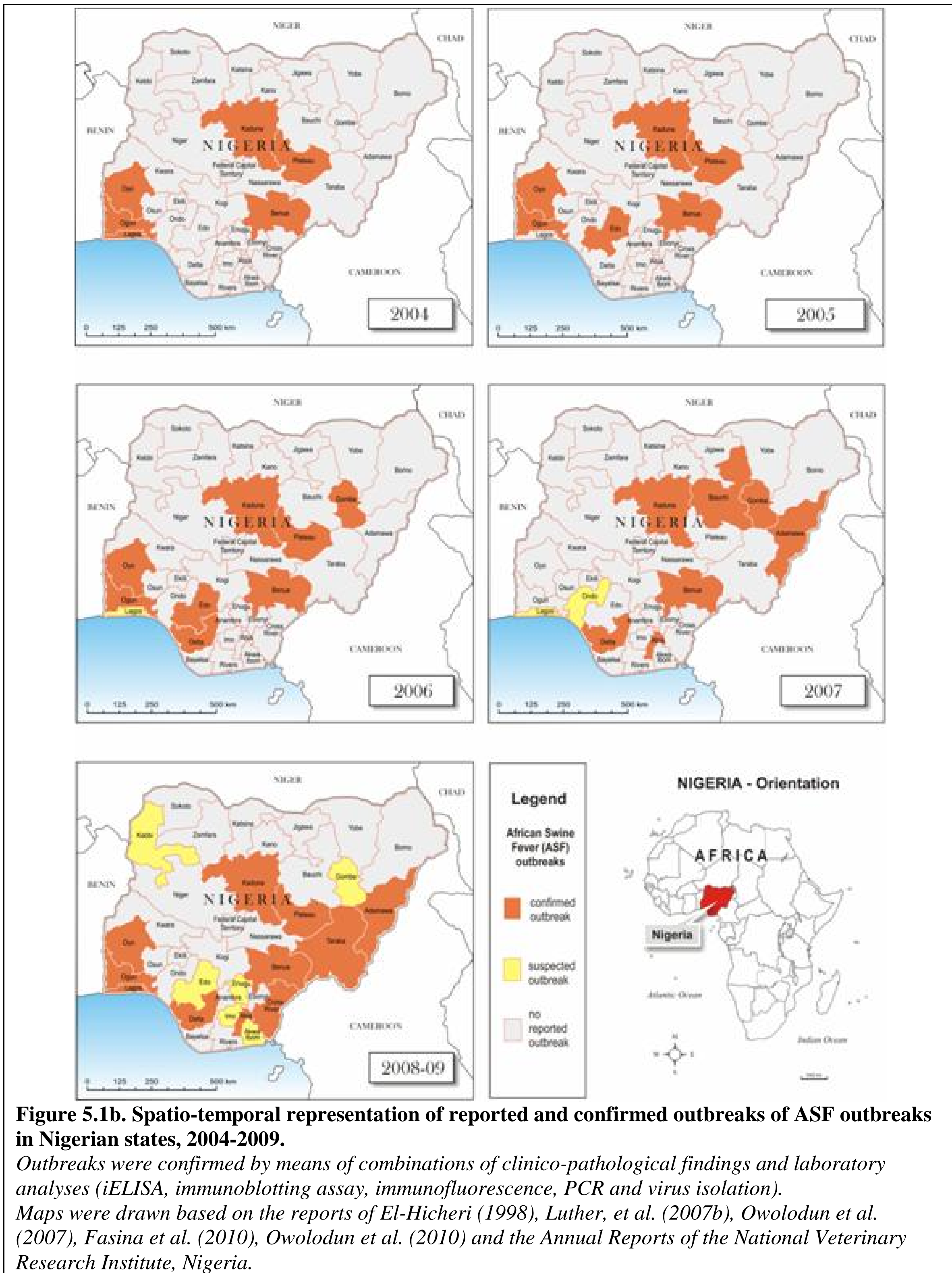


Figure 5.1b. Spatio-temporal representation of reported and confirmed outbreaks of ASF outbreaks in Nigerian states, 2004-2009.

Outbreaks were confirmed by means of combinations of clinico-pathological findings and laboratory analyses (iELISA, immunoblotting assay, immunofluorescence, PCR and virus isolation).

Maps were drawn based on the reports of El-Hicheri (1998), Luther, et al. (2007b), Owolodun et al. (2007), Fasina et al. (2010), Owolodun et al. (2010) and the Annual Reports of the National Veterinary Research Institute, Nigeria.

Table 5.1 Population characteristics of the case and control farms participating in the study of ASF in Nigeria, 2008-2011

Designation	Counts	Herd size							Locations and Descriptions
	Ratio: 1.15:1	Mean±SD	Min.	Max.	25th percentile	50th percentile	75th percentile	95th percentile	
Case	69	98.48±51.77	11	306	65	90	135	180	Selected from a lists of clusters of farms in Imo, Kebbi, Lagos and Taraba
Control	60	95.43±47.96	12	195	52.5	89.5	134	177	Selected from a lists of clusters of farms in Imo, Kebbi, Lagos and Taraba

Data for the risk assessment periods were collected between October 2008 and April 2009 (39 datasets), and February and June 2011 (93 datasets). Several of the large communities of pig farms in Nigeria are grouped in clusters/cooperatives for the purposes of accessing services and marketing facilities jointly.

Table 5.2 Univariable conditional logistic regression analysis of risk factors associated with presence of ASF outbreaks on pig farms, Nigeria, 2008-2011

Variable/risk factor	**Category**	**Cases** ***n*** **(%)**	**Controls** ***n*** **(%)**	***OR***	**95% CI**	***P-value***
Sale method	Community/ abattoir	34 (49.3)	28 (46.7)	1.00	–	–
	Market	35 (50.7)	32 (53.3)	0.82	0.40, 1.65	0.57
Contact infected farm	No	39 (56.5)	27 (45.0)	1.00	–	–
	Yes	30 (43.5)	33 (55.0)	0.64	0.32, 1.27	0.20
Purchased pigs routinely without testing during outbreaks	No	41 (59.42)	43 (71.67)	1.00	–	–
	Yes	28 (40.58)	17 (28.33)	1.72	0.81, 3.66	0.16
Infected neighbourhood	No	22 (31.88)	50 (83.33)	1.00	–	–
	Yes	47 (68.12)	10 (16.67)	8.52	3.81, 19.05	<0.001
Keep other animals on the farm	No	55 (79.71)	53 (88.33)	1.00	–	–
	Yes	14 (20.29)	7 (11.67)	2.18	0.77, 6.19	0.14
Abattoir/slaughter slabs within pig communes	No	14 (20.29)	50 (83.33)	1.00	–	–
	Yes	55 (79.71)	10 (16.67)	20.85	7.80, 55.75	<0.001
Visceral and intestinal contents disposed of indiscriminately	No	23 (33.33)	17 (28.33)	1.00	–	–
	Yes	46 (66.67)	43 (71.67)	1.26	0.59, 2.71	0.55
Wild bird enter pig pens	No	33 (47.83)	46 (76.67)	1.00	–	–
	Yes	36 (52.17)	14 (23.33)	3.57	1.64, 7.76	0.001
Ticks observed on pigs/premises	No	67 (97.10)	55 (91.67)	1.00	–	–
	Yes	2 (2.90)	5 (8.33)	0.39	0.07, 2.23	0.29
Share farm tools with other farms	No	49 (71.01)	13 (21.67)	1.00	–	–
	Yes	20 (28.99)	47 (78.33)	0.11	0.05, 0.26	<0.001
Use treated water	No	44 (63.77)	37 (61.67)	1.00	–	–
	Yes	25 (36.23)	23 (38.33)	0.99	0.48, 2.07	0.99
Rats have access to feed store and pig pens	No	11 (15.94)	30 (50.00)	1.00	–	–
	Yes	58 (84.06)	30 (50.00)	4.77	2.07, 10.97	<0.001
Buy commercial feed/compound	Commercial	14 (20.29)	39 (65.00)	1.00	–	–
	Self milling	55 (79.71)	21 (35.00)	0.14	0.06, 0.31	<0.001

Table 5.3 Multivariable conditional logistic regression analysis of risk factors associated with ASF virus infection in a matched case-control study of pig farms, Nigeria, 2008-2011

Variable/risk factor	Category	OR	95% CI	*P*-value
Infected neighbourhood	No	1.00	–	–
	Yes	3.26	1.20, 8.83	0.02
Abattoir/slaughter slabs in pig communes	No	1.00	–	–
	Yes	8.20	2.73, 24.63	<0.001
Share farm tools with other farms	No	1.00	–	–
	Yes	0.35	0.12, 1.01	0.05

Table 5.4 Univariable conditional logistic regression analysis of the association between self-reported biosecurity practices and the presence of ASF outbreaks on pig farms, Nigeria, 2008-2011

Variable/Biosecurity measure	Category	Case (%)	Control (%)	OR	95% CI	P-value
Restricted access	No	24 (34.78)	14 (23.33)	1.00	–	-
	Yes	45 (65.22)	46 (76.67)	0.48	0.20, 1.11	0.09
Fence around premises	No	23 (33.33)	18 (30.00)	1.00	–	-
	Yes	46 (66.67)	42 (70.00)	0.79	0.37, 1.69	0.54
Gate at entrance	No	25 (36.23)	18 (30.00)	1.00	–	-
	Yes	44 (63.77)	42 (70.00)	0.66	0.30, 1.45	0.30
Foot bath/dips present	No	23 (33.33)	10 (16.67)	1.00	–	-
	Yes	46 (66.67)	50 (83.33)	0.35	0.15, 0.84	0.02
Change solution in foot pans regularly*	No	20 (28.99)	11 (18.33)	1.00	–	-
	Yes	49 (71.01)	49 (81.67)	0.57	0.25, 1.29	0.18
Records kept	No	18 (26.09)	10 (16.67)	1.00	–	-
	Yes	51 (73.91)	50 (83.33)	0.54	0.22, 1.29	0.16
Food and water control	No	28 (40.58)	12 (20.00)	1.00	–	-
	Yes	41 (59.42)	48 (80.00)	0.30	0.13, 0.71	<0.01
Quarantine of newly purchased pigs	No	30 (43.48)	32 (53.33)	1.00	–	-
	Yes	39 (56.52)	28 (46.67)	1.38	0.68, 2.79	0.37
Terminal cleaning (end-of-cycle cleaning)	No	33 (47.83)	20 (33.33)	1.00	–	-
	Yes	36 (52.17)	40 (66.67)	0.52	0.25, 1.08	0.08
Routine cleaning	No	13 (18.84)	12 (20.00)	1.00	–	-
	Yes	56 (81.16)	48 (80.00)	1.08	0.45, 2.59	0.87
Cleaning and disinfection of drinkers and feeders	No	23 (33.33)	28 (46.67)	1.00	–	-
	Yes	46 (66.67)	32 (53.33)	1.60	0.79, 3.23	0.19
Wash/disinfect equipment and tools	No	29 (42.03)	19 (31.67)	1.00	–	-
	Yes	40 (57.97)	41 (68.33)	0.61	0.29, 1.28	0.19
Remove manure and litter routinely	No	15 (21.74)	8 (13.33)	1.00	–	-
	Yes	54 (78.26)	52 (86.67)	0.55	0.22, 1.41	0.22
Prompt disposal of dead animals	No	25 (36.23)	15 (25.00)	1.00	–	-
	Yes	44 (63.77)	45 (75.00)	0.52	0.23, 1.16	0.11
Safe disposal of faeces and carcasses	No	17 (25.00)	8 (13.33)	1.00	–	-
	Yes	51 (75.00)	52 (86.67)	0.42	0.16, 1.10	0.08
Sufficient feeding and drinking space	No	11 (15.94)	9 (15.00)	1.00	–	-
	Yes	58 (84.06)	51 (85.00)	0.81	0.31, 2.12	0.67
Sufficient space for pigs (prevent overcrowding)	No	12 (17.39)	7 (11.67)	1.00	–	-
	Yes	57 (82.61)	53 (88.33)	0.61	0.23, 1.64	0.33
Use disinfectants	No	31 (44.93)	35 (58.33)	1.00	–	-
	Yes	38 (55.07)	25 (41.67)	1.63	0.81, 3.28	0.17
Do not mix pigs of different ages	No	26 (37.68)	11 (18.33)	1.00	–	-
	Yes	43 (62.32)	49 (81.67)	0.32	0.14, 0.75	<0.01
All-in all-out system	No	55 (79.71)	50 (83.33)	1.00	–	-
	Yes	14 (20.29)	10 (16.67)	1.18	0.48, 2.90	0.72
Move from young to old pigs	No	33 (47.83)	18 (30.00)	1.00	–	-
	Yes	36 (52.17)	42 (70.00)	0.48	0.23, 0.98	0.04
Change rubber boots	No	21 (30.43)	14 (23.33)	1.00	–	-
	Yes	48 (69.57)	46 (76.67)	0.58	0.25, 1.32	0.20
Change clothes to go in and out	No	37 (53.62)	29 (48.33)	1.00	–	-
	Yes	32 (46.38)	31 (51.67)	0.78	0.38, 1.58	0.48
Separate/isolate sick pigs	No	22 (31.88)	10 (16.67)	1.00	–	-
	Yes	47 (68.12)	50 (83.33)	0.40	0.17, 0.95	0.04
Consultation and visits of veterinarian/paraveterinarians when animals were sick	No	18 (26.09)	25 (41.67)	1.00	–	-
	Yes	51 (73.91)	35 (58.33)	2.02	0.91, 4.50	0.08
Downtime of >2 weeks	No	41(59.42)	47 (78.33)	1.00	–	-
	Yes	28 (40.58)	13 (21.67)	2.55	1.15, 5.65	0.02
Pest and rodent control	No	33 (47.83)	43 (71.67)	1.00	–	-
	Yes	36 (52.17)	17 (28.33)	2.50	1.20, 5.20	0.02
Evaluate and audit biosecurity measures periodically	No	42 (60.87)	40 (66.67)	1.00	–	-
	Yes	27 (39.13)	20 (33.33)	1.25	0.61, 2.59	0.54

*46 case farms had a foot bath/dip and an additional three farmers used improvised pans in place of a foot dip, making a total of 49.

Table 5.5 Multivariable conditional logistic regression analysis of self-reported biosecurity practices against ASF outbreaks on pig farms, Nigeria, 2008-2011

Variable/Biosecurity measure	Category	OR	95% CI	*P*-value
Food and water control	No	1.00	-	-
	Yes	0.14	0.04, 0.46	<0.001
Separate/isolate sick pigs	No	1.00	-	-
	Yes	0.14	0.04, 0.53	0.004
Consultation and visits of	No	1.00	-	-
veterinarians/paraveterinarians when animals are sick	Yes	8.11	2.13, 30.90	0.002
Wash/disinfect equipment and tools	No	1.00	-	-
	Yes	0.27	0.10, 0.78	0.02
Pest and rodent control	No	1.00	-	-
	Yes	4.94	1.84, 13.29	0.002

5.4 Discussion

In the current study, two sets of factors were studied with regard to the risk of ASF infection on pig farms in Nigeria, namely [A] Farm environment and management practices, and [B] Self-reported biosecurity practices. The former are contributory factors which may predispose farms to a higher risk of infection with the ASF virus while the latter are practices (hygiene and good management) that farmers reported to have taken to reduce the risk of these infections (FAO/OIE, 2010).

[A1]. The presence of an abattoir in pig communities was the risk factor that influenced ASF infection the most (OR = 8.20; $CI_{95\%}$ = 2.73, 24.63; $P < 0.001$). This observation can probably be ascribed to a number of factors, including the following:

1. The farmers tend to present sick and unthrifty pigs for slaughter at abattoirs first, without determining the cause of sickness, some of which may be ASF (Randriamparany et al., 2005; Fasina et al., 2010). Since the ASF virus is present in the tissues and body fluids of slaughtered sick pigs, massive environmental contamination and possible farm infection may result.

2. Rats and wild birds are usually observed near an open abattoir environment. When intestinal content and viscera, which are sometimes infectious, are indiscriminately disposed of, they may be carried to nearby pig farms by these scavengers, thereby facilitating the infection of naïve pigs.
3. Farmers often participate in various processes on abattoir floors with the consequent risk of farm infection.

[A2]. The presence of an infected farm in a neighbourhood was also significantly associated with the infection of farms (OR = 3.26; $CI_{95\%}$ = 1.20, 8.83; P = 0.02). This is related directly to a local spread between and within pig farms and may occur through direct pig-to-pig contact, especially in scavenging populations, by spreading through formites, and possibly by tick vectors (although no tick vector has been associated with ASF in Nigeria to date). Mannelli et al. (1997) and Costard et al. (2009a) have similarly reported that free-range pigs and local pig movement were associated with the spread of ASF in previous studies. Effort must therefore be made to reduce the networks, connectivity and neighbourhood-mediated spread of ASF (Rivas et al., 2010; Firestone et al., 2011).

[A3]. In our analysis, the sharing of tools was marginally negatively associated with the spread of infection (OR = 0.35; $CI_{95\%}$ = 0.12, 1.00; P = 0.05). Since it is logical that tool-sharing may exacerbate the spread of the disease from one location to another, the reason for this observation was not immediately clear. However, in the analysis of the biosecurity measures, the washing/disinfection of tools was also negatively associated with the spread of ASF. It is possible that tools shared between farms are washed and disinfected more often, hence, the negative association (protection) observed. The

practice of sharing tools and equipment will continue for the foreseeable future amongst small scale farmers who may not afford some of the farm equipment.

We evaluated 28 self-reported biosecurity measures. Only five had some association with ASF infection in the final multivariable model. These were food and water control, the separation or isolation of sick pigs, and the washing and disinfection of farm tools and equipment, all of which were negatively associated with ASF seropositivity. Consultation with and visits by veterinarians or paraveterinarians when animals are sick, and pest and rodent control were all positively associated with the risk of seropositivity of pig farms.

[B1]. Food and water control significantly reduced the risk of ASF in this analysis (OR = 0.14; $CI_{95\%}$ = 0.04, 0.46; $P < 0.001$). Since the introduction of food and swill are an important means of transmission of pig diseases (Horst et al., 1997; El-Hicheri, 1998), a carefully planned and isolated feed store and covered water storage remain important parts of a comprehensive biosecurity programme. Such storage facilities also have the advantage of excluding contamination by rodents and wild birds. Contaminated feed and water have played role in the spread of ASF in West Africa in the past (El-Hicheri, 1998).

[B2]. Separation or isolation of sick pigs from healthy ones (OR = 0.14; $CI_{95\%}$ = 0.04, 0.53; $P = 0.004$) was found to be equally important in the prevention of ASF. Infected pigs can shed a large amount of ASF virus, especially naso-pharyngeally, and these viruses may remain in the environment for a long time (FAO, 2009). Hence, the contamination of other pigs is highly likely if an infected pig is retained in the pig herd. Since domestic pig-to-pig contact remains the only proved means of transmission of ASF in Nigeria and West Africa, it is desirable to remove all uninfected pigs from infected/sick pigs to cut off the continued infection of farms.

[B3]. Washing and disinfection of farm equipment and tools was also negatively associated with ASF infection and seropositivity (OR = 0.27; $CI_{95\%}$ = 0.10, 0.78; P = 0.02). Some farm implements are shared between abattoirs and farms, especially in situations where an abattoir is sited inside a pig facility. These implements include shovels, knives, cutlasses, brooms, waste bins, wheelbarrows, etc. Heavily contaminated tools may be returned to the farm without disinfection, and these become sources of infection to naïve pigs.

[B4]. The consultation with and visits of veterinarians or paraveterinarians to farms when animals are sick was positively associated with ASF infection of farms (OR = 8.11; $CI_{95\%}$ = 2.13, 30.90; P = 0.002). There are two possible explanations for this observation. Firstly, farmers usually only call in veterinarians or paraveterinarians when everything else (management procedures and the administration of antibiotics) has failed. The visit of a veterinarian/paraveterinarian is therefore more likely to be a consequence of an ASF outbreak than it is a predisposing factor. It is possible that, during a visit, more animals than those observed as clinically sick are already infected, and these animals will continue to spread infection after the visit unless they are removed alongside the sick animals. Secondly, the course of outbreaks of a disease such as ASF is often a crisis period and a veterinarian/paraveterinarian visiting a cluster of pig farms is likely to visit more than one farm/day in such situation. Tools for the administration of drugs and sampling may be shared, and clothing and shoes may not be changed in-between farms. This inadvertent error may therefore also spread infection to other farms subsequently visited in the course of disease management. Strict observance of biosecurity in-between movement to farms is encouraged by professionals.

[B5]. Finally, pest and rodent control were positively associated with ASF infection on farms in Nigeria (OR = 4.94; $CI_{95\%}$ = 1.84, 13.29; P = 0.002). Farmers do not usually implement/intensify rodent or pest control programmes unless they have problems with these vectors. These rodents/pests may contaminate feed and water, including the pig premises, with remnants taken from abattoir floors, which may predispose farms to ASF infection. Intermittent implementation of pest control programmes may also lead to abnormal local fluctuations in pest populations, which will in turn lead to increased pest movement between farms and a resulting increased risk of disease transmission. Finally, if farmers perceive rodents to be a risk factor for disease, then farmers may implement a rodent control programme in response to an outbreak on or near their farm; therefore the control programme is a consequence of the outbreak rather than of the fact that the presence of rodents is a risk factor.

Spatial and temporal patterns of ASF outbreaks in Nigeria

ASF appears to infect pig farms in Nigeria in a pig trade-related pattern. The outbreaks that started in the Ogun-Lagos axis in 1997-98 were linked to Benin, due to commercial pig-related activities along the border between Nigeria and Benin (El-Hicheri, 1998). Prior to this outbreak, Côte d'Ivoire (1996) and Benin (1997) were infected, and a regional early warning was sent to neighbouring countries to prevent the further spread of infection. However, a porous border, poor veterinary services, legal and illegal trade in pig products across the border and poor disease reporting systems and poor preparation, supported infections and subsequent outbreaks of ASF in Nigeria.

Infection rapidly spread from the Lagos-Ogun axis to some parts of south-west, south-south, south-east and north-central Nigeria, strictly following the trade routes of pigs in the country (El-Hicheri, 1998; Fasina et al., 2009). To date, periodic outbreaks have been found in these locations and pig movement continues to follow the same pattern (see Figures 5.1a & b). Etter et al. (2011) has previously established a similar pattern of infection between Guinea Bissau, Senegal and Gambia – finished pigs are moved north-west to Dakar (a major consumption area), but these pigs are raised adjacent to the enzootic locations of Guinea Bissau and Gambia. Hence, high seroprevalence was obtained in pigs. Thus these trade movements played a critical role in the epidemiology of ASF in that part of West Africa.

Since infection in one area appears to have a contiguous effect on neighbouring areas (see Figures 5.1a & b), it will be important to use a region-based approach to control the spread of infectious diseases such as ASF, in addition to farm-based biosecurity. Such approach will benefit the control of African swine fever and other infectious diseases.

This study was based on the serological and virological results obtained in past surveillance (Fasina et al., 2010). However, because a disease such as ASF is a dynamic system, it will be necessary to determine changes in the epidemiological picture regularly and also to check whether or not the proposed methods are having an impact, using a sustained surveillance system that should itself be evaluated periodically. The continuing surveillance and evaluation of risk factors supporting infection of pig farms in West Africa remains the key component in the development of a risk-based approach to understanding the epidemiology of ASF in the sub-region (Etter et al., 2011).

Biosecurity is a set of measures that are interlinked with one another and with good husbandry/management practices. Husbandry practices and management styles used on farms should be evaluated to determine good hygiene practices that will suit Nigerian-type piggeries (Costard et al., 2009b).

Our study was subject to a number of limitations, including the possibility of some types of bias. Every effort was made to reduce confounding bias by

- matching for farm population types and locations;
- restricting the study period and area to reduce model bias and confounding factors; and
- using a multivariable conditional logistic regression model to control for confounding factors between the measured predictors.

Self-report bias was another potential source of error in the study. We are aware that farmers may have wanted to give “socially acceptable” responses to the questions and that the level of self-reported biosecurity may have been at variance with the actual implementation, as reported in past studies (Nespeca et al., 1997; Casal et al., 2007). However, where possible, we observed farm management and biosecurity practices which were static and straightforward (such as fences, restricted access, records, disposal pits, abattoirs), and used them as a check against questionnaire responses, as recommended by Stark et al. (1998).

In addition, the concept of biosecurity may have different interpretations to different farmers in terms of its comprehensiveness and content, but this lack of precision was addressed and reduced through the open fora and large group discussions held before the administration of questionnaires, where some agreement was reached. In this study, the

problem of recall bias was considered to be negligible, since ASF was an ongoing infection in almost all the case farms selected for the study, and the control farms were fully aware of its presence. Spatial bias was also managed further by matching location. The selection of control farms around the case farms was random.

This study avoided the use of face-to-face interviews, because we wanted to eliminate professional bias, a situation where the interviewer's own concept of biosecurity (because of professional training) might be passed on and could influence the farmers' answers. The problems that may be associated with unsupervised questionnaire administration were minimized by the use of simple and unambiguous questions, pre-administration discussions and closed-ended questions.

5.5. Conclusions

The following conclusions have been reached in this study:

It is likely that the presence of an infected pig farm in the same neighbourhood and the presence of abattoirs and associated practices will increase the likelihood of ASF infection of farms. This also applies to the presence of vermin and wild birds in the pig farm community. However, strict food and water control, the immediate separation (isolation) of sick pigs from healthy pigs, and the washing and/or disinfection of farm equipment will assist in reducing the chances of infection. Region-based control of infectious diseases, together with farm-based biosecurity, will assist in controlling future outbreaks of ASF in Nigeria and West Africa.

Acknowledgements

The authors wish to thank Mrs Ingrid Booysen, Department of Geography, Geoinformatics and Meteorology, University of Pretoria, for the maps, Ms Idette Noomé, Department of English, University of Pretoria for English revision and also the farmers who answered the questionnaire for their kind assistance.

5.6. References

Bastos, A.D.S., Penrith, M.-L., Crucière, C., Edrich, J., Hutchings, G., Roger, F., Couacy-Hymann, E., Thomson, G.R., 2003. Genotyping field strains of African swine fever virus by partial *p72* gene characterization. Archives Virol. 148, 693–706.

Bastos, A.D.S., Penrith, M.-L., Macome, F., Pinto, F., Thomson, G.R., 2004. Co-circulation of two genetically distinct viruses in an outbreak of African swine fever in Mozambique: no evidence for individual co-infection. Vet. Microbiol. 103, 169–182.

Boshoff, C.I., Bastos, A.D.S., Gerber, L.J., Vosloo, W., 2007. Genetic characterisation of African swine fever viruses from outbreaks in southern Africa (1973-1999). Vet. Microbiol. 121, 45–55.

Casal, J., de Manuel, A., Mateu, E., Martin M., 2007. Biosecurity measures on swine farms in Spain: perceptions by farmers and their relationship to current on-farm measures. Prev. Vet. Med. 82, 138–150.

Costard, S., Porphyre, V., Messad, S., Rakotondrahanta, S., Vidon, H., Roger, F., Pfeiffer, D.U., 2009a. Multivariate analysis of management and biosecurity practices in smallholder pig farms in Madagascar. Prev. Vet. Med. 92, 199–209.

Costard, S., Weiland, B., de Glanville, W., Jori, F., Rowlands, R., Vosloo, W., Roger, F., Pfeiffer, D.U., Dixon, L.K., 2009b. African swine fever: how can global spread be prevented? Phil. Transact. Royal Society B 364, 2683-2696.

El-Hicheri, K., 1998. Emergency assistance on control and eradication of an outbreak of African swine fever in Western Nigeria. *Report of the FAO Consultancy Mission to Nigeria.* TCP/NIR/7822(E). FAO, Rome, December.

Etter, E.M.C., Seck, I., Grosbois, V., Jori, F., Blanco, E., Vial, L., Akakpo, A.J., Bada-Alhambedji, R., Kone, P., Roger, F.L., 2011. Seroprevalence of African swine fever in Senegal, 2006. Emerg. Infect. Dis. 17(1), 49–54.

Fasina, F.O., Shamaki, D., Makinde, A.A., Pitt, A.S.F., 2009. Field surveillance and laboratory diagnoses of African swine fever in Nigeria (IAEA-CN-174-151). Book of Synopses, FAO/IAEA International Symposium on Sustainable Improvement of Animal Production and Health, 8-11 June 2009, Vienna, Austria, pp. 301-302.

Fasina, F.O., Shamaki, D., Makinde, A.A., Lombin, L.H., Lazarus, D.D., Rufai, S.A., Adamu, S.S., Agom, D., Pelayo, V., Soler, A., Simón, A., Adedeji, A.J., Yakubu, M.B., Mantip, S., Benshak, A.J., Okeke, I., Anagor, P., Mandeng, D. C., Akanbi, B.O., Ajibade, A.A., Faramade, I., Kazeem, M.M., Enurah, L.U., Bishop, R., Anchuelo, R., Martin, J.H., Gallardo, C., 2010. Surveillance for African swine fever in Nigeria, 2006-2009. Transboundary Emerg. Dis. 57(4), 244–253.

Firestone, S.M., Schemann, K.A., Toribio, J-A.L.M.L., Ward, M.P., Dhand, N.K., 2011. A case-control study of risk factors for equine influenza spread onto horse premises during the 2007 epidemic in Australia. Prev. Vet. Med. 100, 53–63.

Food and Agricultural Organization of the United Nations (FAO), 2009. African swine fever spread in the Russian Federation and the risk for the region. EMPRES WATCH, December 2009. Available online at: ftp://ftp.fao.org/docrep/fao/012/ak718e/ak718e00.pdf. Accessed on 6th March, 2010.

Food and Agricultural Organization of the United Nations (FAO) and World Organization for Animal Health (OIE), 2010. Good Practices for Biosecurity in the Pig Sector: Issues and Options in Developing and Transition Countries. FAO Animal Production and Health Paper no. 169. Available at http://www.fao.org/docrep/012/i1435e/i1435e00.pdf. Accessed on 20 August 2011.

Gallardo, C., Mwaengo, D.M., Macharia, J.M., Arias, M., Taracha, E.A., Soler, A., Okoth, E., Martín, E., Kasiti, J., Bishop, R.P. 2009. Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the *p54*, *p72*, and *pB602L* (*CVR*) genes. Virus Genes 38(1), 85–95.

Gallardo, C., Ademun, A.R., Nieto, R., Nantima, N., Arias, M., Martin, E., Pelayo, V., Bishop, R.P., 2011. Genotyping of African swine fever virus (ASFV) isolates associated with disease outbreaks in Uganda in 2007. African J. Biotechnol. 10(17), 3488–3497.

Horst, H.S., Huirne, R.R., Dijkhuizen, A.A., 1997. Risks and economic consequences of introducing classical swine fever into The Netherlands by feeding swill to swine. Rev. Sci. Tech. 16, 207–214.

Hosmer, D.W., Lemeshow, S., 1989. Logistic regression for matched case-control studies. In: Hosmer, D.W., Lemeshow, S. (Eds.), Applied Logistic Regression. Wiley, New York, pp. 187–215.

Lubisi, B.A., Bastos, A.D., Dwarka, R.M., Vosloo, W., 2005. Molecular epidemiology of African swine fever in East Africa. Arch. Virol. 150(12), 2439–2452.

Lubisi, B.A., Bastos, A.D., Dwarka, R.M., Vosloo, W., 2007. Intra-genotypic resolution of African swine fever viruses from an East African domestic pig cycle: a combined *p72-CVR* approach. Virus Genes 35(3), 729–735.

Luther, N.J., Majiyagbe, K.A., Shamaki, D., Lombin, L.H., Antiabong, J.F., Bitrus, Y., Owolodun, O., 2007a. Detection of African swine fever virus genomic DNA in a Nigerian Red River hog (*Potamochoerus porcus*). Vet. Rec. 160, 58–59.

Luther, N.J., Shamaki, D., Majiyagbe, K.A., Nwosuh, C.I., 2007b. The prevalence of African swine fever in parts of the middle belt central states of Nigeria. Bulletin Anim. Prod. Hlth. Africa 55, 79–81.

Mannelli, A., Sotgia, S., Patta, C., Sarria, A., Madrau, P., Sanna, L., Firinu, A., Laddomada, A., 1997. Effect of husbandry methods on seropositivity to African swine fever virus in Sardinian swine herds. Prev. Vet. Med. 32(3-4), 235–241.

Nespeca, R., Vaillancourt, J-P., Morrow, W.E.M., 1997. Validation of a poultry biosecurity survey. Prev. Vet. Med., 31, 73–86.

OIE – see World Organisation for Animal Health

Owolodun, O.A., Yakubu, B., Antiabong, J.F., Adefalujo, O.K., Ogedengbe, M.E., Obisakin, E.T., Shamaki, D., 2007. Molecular assessment of African swine fever in North-Central Nigeria. Bulletin Anim. Prod. Hlth. Africa 55, 96–103.

Owolodun, O.A., Bastos, A.D.S., Antiabong, J.F., Ogedengbe, M.E., Ekong, P.S., Yakubu, B., 2010. Molecular characterisation of African swine fever viruses from Nigeria (2003–2006) recovers multiple virus variants and reaffirms CVR epidemiological utility. Virus Genes 41, 361-368. DOI 10.1007/s11262-009-0444-0.

Penrith, M-L., Thomson, G.R., Bastos, A.D.S., 2004. African swine fever. In: Coetzer, J.A.W., Turstin, R.C. (Eds.), Infectious Diseases of Livestock, 2nd edition, Oxford University Press, Cape Town, pp. 1088–1119.

Randriamparany, T., Grenier, A., Tourette, I., Maharavo-Rahantamala, C.Y., Rousset, D., Lancelot, R., 2005. Epidemiological situation of African swine fever in Lake Alaotra Region (Madagascar) and possible consequences on the organization of disease control and surveillance. Rev. Elev. Med. Vet. Pays Trop. 58, 15–20.

Rivas, A.L., Chowell, G., Schwager, S.J., Fasina, F.O., Hoogesteijn, A.L., Smith, S.D., Bisschop, S.P.R., Anderson, K.L., 2010. Lessons from Nigeria: the role of roads in the geo-temporal progression of the avian influenza (H5N1). Epid. Inf. 138, 192–198.

Rowlands, R.J., Michaud, V., Heath, L., Hutchings, G., Oura, C., Vosloo, W., Dwarka, R., Onashvili, T., Albina, E., Dixon, L.K., 2008. African swine fever virus isolate, Georgia, 2007. Emerg. Infect. Dis. 14, 1870–1874.

Stärk, K., Pfeiffer, D., Morris, R., 1998. Risk factors for respiratory diseases in New Zealand pig herds. NZ Vet. J. 46, 3–10.

Vial, L., Weiland, B., Jori, F., Etter, E., Dixon, L., Roger, F., 2007. African swine fever virus DNA in soft ticks, Senegal. Emerg. Infect. Dis. 13, 1928–1931.

World Organisation for Animal Health (OIE), 2008. African swine fever. In: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees). Chapter 2.8.1; Web (Sixth) edition. Available at http://www.oie.int/eng/normes/mmanual/2008/pdf/2.08.01_ASF.pdf. Accessed on 20th January, 2009.

World Organisation for Animal Health (OIE), 2011. WAHID Interface Animal Health Information (African swine fever). Available at: http://web.oie.int/wahis/public.php. Accessed on 20th June, 2011.

6.0 PHYTOCHEMICAL ANALYSIS AND *IN VITRO* ANTI-AFRICAN SWINE FEVER VIRUS ACTIVITY OF EXTRACTS AND FRACTIONS OF *ANCISTROCLADUS KORUNPENSIS*, THOMAS AND GEREAU (ANCISTROCLADACEAE)

Folorunso O. Fasina[1,2], Oyinlola O. Olaokun[3], Olusola O. Oladipo[4], Margaret M. Fasina[5], Adesoji A. Makinde[4], Livio Heath[6], Amanda D. S. Bastos[2]

Folorunso O. Fasina[1,2]
Email: daydupe2003@yahoo.co.uk ; dayo.fasina@up.ac.za

Oyinlola O. Olaokun[3]
Email: oyinolaokun@yahoo.com

Olusola O. Oladipo[4]
Email: lekan529@yahoo.com

Margaret M. Fasina[5]
Email: modupeday2005@yahoo.co.uk

Adesoji A. Makinde[4]
Email: sojiomak@yahoo.com

Livio Heath[6]
Email: HeathL@arc.agric.za

Amanda D. S. Bastos[2]
ADBastos@zoology.up.ac.za

[1] Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa.

[2] Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, South Africa.

[3] Phytomedicine Programme, Department of Paraclinical Science, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa.

[4] National Veterinary Research Institute, Vom, Plateau State, Nigeria.

[5] Maximum Farms, P. O. Box 23, Vom Post Office, Plateau State, Nigeria.

[6] Transboundary Animal Disease Programme, ARC-Onderstepoort Veterinary Institute, Onderstepoort, South Africa.

Aim: *To verify farmers' claims of effectiveness of certain plants against African swine fever based on various ethnoveterinary treatment protocols during past outbreaks.*

6.0.1 ABSTRACT

Background

African swine fever (ASF), a highly contagious fatal acute haemorrhagic viral disease of pigs currently has no treatment or vaccination protocol and it threatens the pig industry worldwide. Recent outbreaks were managed by farmers with ethnoveterinary preparations with various claims of effectiveness.

Methods

In this work, we determined certain compounds from the plant *Ancistrocladus korupensis* and explore the *in-vitro* antiviral potentials of extracts and fractions of extract of *Ancistrocladus korupensis* against African swine fever virus using the primary cell culture and PCR assays.

Results

We identified 35 compounds using GC-MS protocol and ASF virus (NIG 99) genome was significantly reduced by some extracts and fractions of the plant. However, the plant was poorly extracted by water and cytotoxicity was found to be a major problem with the use of the plant since its extracts also reduced the viability of the primary cells used in the assay.

Conclusion

It is confirmed that the plant has antiviral potentials against ASF virus and farmers' claims seem to have certain degree of veracity, but finding the best means of exploring the potential of the plant while reducing its cytotoxic effect *in-vitro* and *in-vivo* will be necessary.

Keywords: *Ancistrocladus korupensis*, African swine fever virus, antiviral,

6.1. BACKGROUND

African swine fever (ASF), a highly contagious fatal acute haemorrhagic viral disease of pigs results in major economic losses and has substantial food security implications. The disease continues to devastate animal resources (pigs) in parts of Sub-Saharan Africa and other infected regions of the world [1,2]. Though studies are on-going with regards to the preventative actions and immunology of ASF virus (ASFV), to date, little success has been made with regards to the development of preventive vaccine targeting the ASF virus or an effective treatment [3]. This is due to the complex nature of the virus, the acute fatality associated with it and the lack of deep understanding of the immune response in ASF infection [4].

Currently, in the event of an outbreak and its possible spread within pig herds, the standard practice remains the zoning, culling of the herd (stamping out) and payment of compensation to prevent epizootics alongside zoosanitary/biosecurity measures [1], However, in most African countries, the lack of subsidies for animal agriculture and poor implementation of compensation policy has negatively impacted prompt reporting and weakened transboundary animal disease control. Alternative and complementary therapies have instead been explored by resource-poor small-scale pig farmers in West Africa, in an attempt to save their stock in an outbreak situation. This has included unorthodox methods, including the use of plants and other ethnoveterinary preparations, with widely ranging claims of effectiveness.

Medicinal plants have been used as remedies for centuries and numerous ethnoveterinary assessments of Nigerian/West African plants have been undertaken to evaluate their effectiveness [5-9]. The effectiveness of plant products used as antiviral agents are well-described [10,11]. Many plants have been used by farmers to 'manage' ASF, however, there are limited peer-reviewed records of plants in general,

and none from West Africa, that have evaluated activity against ASF virus [12-14]. One such plant fed to pigs has had unconfirmed reports of reducing morbidity and mortality, and there have even been claims of complete recovery following oral administration of *Ancistrocladus korupensis* preparations. This Liana plant species was recently described by Thomas and Gereau [15] and is mainly found in the tropical swamp of the Korup National Park in Cameroun and adjoining Cross River National Park in Nigeria. It has a low population density, with approximately 13 plants per hectare [16].

Anti-malarial and anti-HIV properties of the plant have been reported [17-20]. In particular, certain naphthylisoquinoline alkaloids (including korundamine, yaoundamine, korupensamine) have been shown to have a wide range of anti-HIV, antimalarial, fungicidal, larvicidal and moluscicidal biological activities [17-19]. Michellamine B, another alkaloid found in the plant, has demonstrated anti-HIV activity through inhibition of viral reproduction, syncitium formation, enzymatic and cell killing activities [18, 20]. However, to date, no assessment of this plant material has been carried out against the ASF virus affecting pigs or for any other animal virus. In this study, we used a molecular biology approach to evaluate the antiviral potentials of this plant in an *in-vitro* model of infection. Primary bone marrow cells were infected with ASFV, treated with crude extracts and fractions of *A. korupensis* and their effect on the virus evaluated by real-time and conventional PCR.

6.2. METHODS

6.2.1 Pre-screening of ethnoveterinary products used for ASF management, plant collection and identification.

Oral interviews were conducted with selected pig farmers from different zones of Nigeria on the management of African swine fever using ethnoveterinary preparations during the past outbreaks of the disease, as part of the national swine disease surveillance programme. Many ethnoveterinary preparations were reported as being used which were screened against published resources to determine whether any preliminary antiviral potential of the plants on the list, had been confirmed [6,9]. *Ancistrocladus korupensis* was selected on this basis, for further assessment against the African swine fever virus.

The plant was identified at the Cross River National Park, and sample specimens were collected for the preparation of a herbarium. Authentication of the plant was carried out at the Herbarium of the Federal College of Forestry, Jos, Nigeria using the standards of Thomas and Gereau [15], and deposited in the herbarium. The plants were air-dried in the laboratory and separated into portions of leaves, stems and roots. Each portion was pulverised using Jika-Werke M20 blender (Jika-Werke, Staufen, Germany) and stored in airtight cellophane bags at +4°C until used.

6.2.2 Phytochemical screening of leaves, stem and roots of Ancistrocladus korupensis

Portions of the pulverised plant were screened for phytochemical content and for certain secondary metabolites including alkaloids, flavonoids, cardiac glycosides, saponins, tannins, anthraquinones, triterpenes and steroids using standard methods [5, 21-23].

6.2.3 Determination of chemical compounds from A. korupensis

The chemical compounds present in the plant were determined using the analysis of gas chromatography-mass spectrometry (GC-MS) and the modified method of Ivanov and Sandell [24]. Briefly described, 2g portions of leaves, stems and whole plant (stems, leaves and roots) of *A. korupensis* were each extracted with petroleum ether and injected into column of the Shimadzu Gas Chromatograph-Mass Spectrometer (GC-MS) QP 2010 PLUS (Shimadzu, Japan) and its software programme for analysis. Oven temperature was 60°C and injection temperature was 250°C, with a linear velocity of 46.3 cm/sec, a capillary column flow rate of 1.61 mL/min and a pressure of 100.2 kPa. For the GC programme, the Ion source was set at 200°C and the interface temperature of 250°C had a threshold of 3000 [25]. The MS analysis was done based on comparative retention times, mass and peaks of the chemical compounds using the NIST05.LIB as the reference database [26]. This library enables the facilitation of comparison of generated spectra with the standards using Probability Based Matching algorithms [26].

The (GC-MS) QP 2010 PLUS had also been pre-fitted with a set of automated internal validity programmes for the analysis, including the adjustment of retention time function, scan measurement, quick and accurate compound identification from chromatogram, search based on mass spectra similarity and other quality assurance-quality control functions [27].

6.2.4 Extractions and Fractions from A. korupensis

Individual distilled water, Acetone (Ace), Methanol (MeOH), Hexane (Hex), Chloroform (Chloro) and DiChloroMethane (DCM) extracts of the plant parts were made separately using previously described methods [28]. Briefly described, 10g of

the finely grounded plant material was soaked in 100ml of each of the solvents in different Erlenmeyer flasks. The contents were shaken on a shaker (Labotec Pty, South Africa) for 30 minutes after which each was spun at ≈2150rpm for 5 minutes in a Rotofix 32A centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). The supernatants were filtered using a 125mmØ No. 1 Whatman filter paper and a glass funnel into pre-weighed glass vials. The whole process was repeated thrice for each extract to exhaustively extract the plant materials and the total volumes of each filtrate were combined and solvent air-dried at room temperature in a fume cupboard. The final products were weighed individually and stored at +4°C until used.

The different extracts were dissolved in dimethyl sulfoxide (DMSO) to make a final concentration of 100mg/ml stock solution (ratio of 100mg of extract to 1ml DMSO). Unfortunately, the quantity of materials recovered from the water extraction process was insignificant to enable further analysis.

6.2.5 Phytochemical Analysis on silica gel

Portions (1ml) of the acetone extract were dissolved in 9ml of Hexane, Dichloromethane, Acetone and Methanol to make a 10 mg/ml fractions of each solution. Ten microlitres (10µl) of each solution was spotted on a pre-labelled aluminium-backed thin-layer chromatographic (TLC) silica plates (TLC Silica Gel 60 F_{254}, Merck, Darmstadt, Germany) with a micropipette, 1ml from the bottom of the plates and thin-layer-chromatography was carried out in Ethyl acetate-methanol-water [40:5.4:5] (EMW; polar/neutral), Benzene-Ethanol-Ammonia [90:10:1] (BEA; non-polar/basic), and Chloroform-Ethyl acetate-Formic acid [5:4:1] (CEF; intermediate polarity/acidic) using the method of Kotze and Eloff [28]. Chromatograms were developed in closed tanks in which the eluent wetted the TLC plates. The final chromatograms were air-dried and sprayed with Vanillin vapour (0.1g)-Methanol

(28ml)-H_2SO_4 (1ml) solution. The Vanillin-sprayed plates were then heated with dry heat for approximately 3 minutes at 110°C for optimal colour development and detection of the separated compounds.

Based on the expression on BEA, the retention factors of the 10 clearly identified compounds were calculated using the formula:

$$R_f = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent}}$$

6.2.6 African swine fever virus and the Primary bone marrow culture.

ASF NIG/99 (a haemadsorbing virus responsible for major ASF outbreaks in Nigeria in 1999) was obtained from the virus repository of the Transboundary Animal Disease Programme (TADP) of the ARC-Onderstepoort Veterinary Institute, South Africa. Primary bone marrow culture (PBMC) adjusted to 1 x 10^7 cells/ml was prepared in the 96-well flat bottom tissue culture plates (Corning Costar®, Sigma Aldrich, Aston Manor, South Africa) according to the standard procedures (Carrascosa et al., 2011), and incubated at 37°C for 48 hours at 5% CO_2. The plates were observed under the microscope for growth of macrophages, after which the liquid contents of the plates were discarded 48 hours post preparation and 100µl of freshly prepared growth medium was dispensed into each well of the plates. The primary cells were then available for virus infection.

6.2.7 Cell viability and cytotoxicity assays.

To assess for the viability of the PBM cells, consistency of the plates were checked for colour change (light orange to pale yellow due to active metabolism and acidification in the plates). Furthermore, each culture plate was inoculated with 100µl of ASF NIG/99 virus (7.0 log_{10} HAD_{50}/ml.) and the placebo (wash buffer), sealed and

incubated in a 5% CO_2 incubator at 37°C for 48 hours and checked for haemadsorption activity (rosette formation) and cytopathic effect (CPE). Cytotoxicity assay was done by inoculating the PBMC with different concentrations of crude acetone extract of *A. korupensis* (1000mg/ml, 500mg/ml, 100mg/ml, 50mg/ml, 25mg/ml, 5mg/ml and 1 mg/ml), and the diluent (DMSO); and checking daily for decreasing number of macrophages and rosette formations.

6.2.8 Antiviral Assays of extract of A. korupensis and its fractions on African swine fever

6.2.8.1 Cell culture Assay system

The modified methods of Vanden Berghe et al., [10] and Ying-Wang et al., [11] were used to carry out the antiviral assessment of the plant.

Fresh PBMCs were prepared on the 96-well flat bottom tissue culture plates as stated above and seeded with 100µl of the ASF NIG/99 virus (7.0 $\log_{10}$ HAD_{50}/ml).

One in two (1:2) serial dilutions of the extracts (Acetone, Hexane, DochloroMethane, Methanol and Chloroform) and acetone fractions (Hexane, Methanol, Ethyl Acetate and Chloroform) were prepared in ordinary 96-well U-bottom plates to deliver 1mg/ml up to 0.0078mg/ml in a 50µl of each dilution. These dilutions were added to rows in the ASF infected plates immediately (see Table 6.1). *Ficus lutea* extracts were used as plant controls. Only 50µl of the wash buffer was added to the positive controls and no virus, extract or fraction was added to the negative controls. The plates, prepared in triplicate were sealed, and each of the experiments was performed in duplicate. The plates were incubated in a 5% CO_2 incubator at 37°C for 48 hours and checked for haemadsorption activity (rosette formation) and CPE.

Table 6.1. Set up of the test system for the antiviral assay

	1	2	3	4	5	6	7	8	9	10	11	12
mg/ml	ASF NIG/99 (+)	Acetone (C)	Hexane(C)	DCM (C)	Chloroform (C)	Methanol (C)	Hexane (F)	Methanol (F)	Ethyl acetate (F)	Chloroform (F)	Blank	Placebo (-)
1	+	*	*	*	*	*	*	*	*	*		-
0.5	+	*	*	*	*	*	*	*	*	*		-
0.25	+	*	*	*	*	*	*	*	*	*		-
0.125	+	*	*	*	*	*	*	*	*	*		-
0.0625	+	*	*	*	*	*	*	*	*	*		-
0.03125	+	*	*	*	*	*	*	*	*	*		-
0.015625	+	*	*	*	*	*	*	*	*	*		-
0.0078125	+	*	*	*	*	*	*	*	*	*		-

(C) = crude extract; (F) = Fraction; DCM = Dichloro Methane; + = Positive control (ASF NIG/99); * = Crude extract or fractions as stated in the table; - = Placebo/Negative control (wash buffer)

6.2.8.2 PCR and Real-time PCR

Following a 7-day incubation period, the plates were observed under the microscope and the 1mg/ml test systems of the *A. korupensis* extracts and their fractions were harvested and assessed by conventional PCR targeting a 478 bp region of the *p72* gene to determine if there was any reduction in viral titres due to the effect of the plant. Briefly, viral DNA was extracted from the harvests using the supplier-prescribed *High Pure PCR Template Preparation Kit* (Roche Diagnostic GmbH, Mannheim, Germany) protocol. A set of forward and reverse primers: (p72U-5′-GGCACAAGTTCGGACATGT-3′ [sense] and p72D- 5′-TGTAACGCAGCACAG-3′ [anti-sense] were used to amplify the target genes at the C-terminal end of virus protein (VP) 72, as previously described [29]. The resulting products were sized by 1.5% agarose gel electrophoresis against a 100bp marker (Fermentas).

Real time/quantitative PCR (qPCR) was used to determine the residual quantity of the ASF viral genome that was left in the extract/fraction treated samples. Briefly, a set of forward (King-s (5′-CTGCTCATGGTATCAATCTTATCGA-3′) and reverse King-a (5′-GATACCACAAGATCRGCCGT-3′) primers, that amplify a conserved 250 bp region of VP72 gene were combined with a TaqMan probe (5′-FAM-

CCACGGGAGGAATACCAACCCAGTG-TAMRA-3′) that detects the amplified product with the label/reporter at the 5′ end [6-carboxy-fluorescein (FAM) and a quencher at the 3′ end (6-carboxy-tetramethyl-rhodamine (TAMRA)] [30]. The system was optimised at 95°C for 3 min; 95°C for 10s; 58°C for 30s and 45 cycles with a cycle threshold (Ct) value of 32±2. The complete protocol is available at http://asf-referencelab.info/asf/files/SOPs/SOP-ASFPCR22008.pdf (Appendix 6).

6.2.8.3 Re-infectivity Assay

Re-infectivity assay was performed to determine whether the observed effect of the plant on the virus was virucidal or virustatic and to correlate the PCR results with the cell culture; briefly, 100µl of the recently harvested virus-extracts/fractions as well as the positive (ASF NIG/99 virus) and the negative (wash buffer) controls were filtered using the 0.22µ filter and inoculated again into freshly prepared PBMC. The culture plates were incubated as described above and microscopically inspected 72, 96 and 120 hours post-infection to determine the residual virus infectivity following exposure to the extracts/fractions.

6.3. RESULTS

6.3.1 Phytochemical screening of leaves, stem and roots of A. korupensis

Leaf, root and stem portions of the pulverised plant revealed the presence of alkaloids, cardiac glycosides and steroids; saponins and flavonoids were only recovered from the leaves while tannins were recovered from the stem (Table 6.2). None of the plant portion contained anthraquinones.

Table 6.2: Secondary metabolites found in the different stem barks, leaves and roots of the *A. korupensis*

Sample	Saponin	Alkaloids	Cardiac glycosides	Steroids	Tannin	Anthraquinones	Flavonoids
Leaves	+	+	+	+	-	-	+
Roots	-	+	+	+	-	-	-
Stem	-	+	+	+	+	-	-

6.3.2 Determination of chemical compounds from A. korupensis

The chemical compounds present in the different portions of the plants identified by Gas Chromatography-Mass Spectrometry (GC-MS) are summarised in Table 6.3. A total of 35 chemical compounds were identified with N-Formylkorupensamin B being the most abundant in the plant but concentrated more in the stem and leaves. Certain compounds or their derivatives were present in all parts of the plant while other compounds were recovered only from certain parts of the plant (see Table 6.3, and appendix D).

Table 6.3. Compound expressed from the stem barks, leaves and roots of *Ancistrocladus korupensis*.

	Compounds (source)*	Molecular weight	Retention time	Retention index
1.	n-Hexadecanoic acid (S, L)	256	25.43	1968
2.	7-Hexadecenoic acid (S)	268	27.24	1886
3.	9-Hexadecenoic acid (S, L, R)	254	27.76	1976
4.	Octadecanoic acid (S, L)	284	28.06	2167
5.	1-Butanamine (S)	155	29.58	1103
6.	1,9-Nonanediol (S, R)	160	31.63	1401
7.	Hexadecanoic acid (S, L, R)	330	31.94	2498
8.	3-Bromooctane (S)	192	32.37	1049
9.	1,3-Tetradecenal (S)	210	33.88	1591
10.	2-Quinolinecarboxylic acid (S)	421	38.31	2310
11.	N,N′-Bis (p-Methoxybenxylidine) benzidine (S, L, R)	420	38.50	3749
12.	N-Formylkorupensamin b (S, L)	407	39.28	3792
13.	4-Acetoxy-6′,7-dimethyl-5′,8′-dimethoxy-1,2′-binaphthalene-1′,4′,5,8-tetrone (S)	460	41.97	3926
14.	4-Pentadecyne (S)	242	42.69	1755
15.	Stigmasterol,22,23-dihydro- (S)	414	44.14	2731
16.	Bicyclo[3.1.0]hexan-3-ol (S)	154	45.35	1079
17.	(Z)6,(Z)9-Pentadecadien-1-ol (L)	224	31.56	1771
18.	13-Oxabicyclo[10.1.0]tridecane (L)	182	33.88	1450
19.	7-Tetradecenal (L)	210	31.63	1609
20.	Squalene (L, R)	410	35.58	2914
21.	Silane (L)	442	35.78	2647
22.	Beta-Tocopherol (L, R)	416	38.30	3036
23.	1H,3H-Furo[3,4-c]furan (L, R)	446	39.28	3243
24.	Vitamin E (dl-alpha-Tocopherol) (L, R)	430	39.96	3149
25.	Gamma-Sitosterol (L)	414	44.14	2731
26.	3,6-Octadien-1-ol,3,7-dimethyl-(Z)- (L)	154	45.37	1228
27.	Cyclopentanol,3-methyl-2-(2-pentenyl)- (L)	168	46.09	1315
28.	Decane, 1-chloro- Decyl Chloride (R)	176	44.13	1240
29.	3-Octadecyne (R)	250	45.34	1828
30.	2-Isopropyl-5-methylcyclohexymethanol (R)	170	42.68	1280
31.	9-Octadecenoic acid (R)	296	27.24	2085
32.	1-Fluorononane (R)	146	31.93	889
33.	3,8-Dibenzoyl-1-nitro-3,6,8-triazabicyclo[4.3.1]decane (R)	394	32.83	3353
34.	Oxalic acid (R)	368	28.68	2606
35.	2,2,3,3,4,4,-Hexamethyltetrahydrofuran (R)	156	29.58	992

*Source of chemical compound as determined by gas chromatograph-mass spectrometry. S = stem, L = leaves and R = combination of residual leaves, stem and root. A total of 35 compound and its derivatives were clearly isolated from the *A. korupensis* stem, leaves and root.

See appendix E 1-3 for details of the identified compounds.

6.3.3 Phytochemical constituents expressed on silica gel.

Thin-layer-chromatography of fractions of Acetone extracts in Ethyl acetate-methanol-water (EMW), Benzene-Ethanol-Ammonia (BEA), and Chloroform-Ethyl acetate-Formic acid (CEF) revealed that several active principles exist in *A. korupensis* and that these were best expressed using BEA followed by CEF and then

EMW. It appears that the dominant principles in the plant were non-polar/basic compounds but other chemicals with varying polarities were also observed. The retention factors (R_f) of the 10 clearly identified compounds in BEA were: 0.125; 0.175; 0.225; 0.263; 0.375; 0.538; 0.763; 0.850; 0.888 and 0.925 (see figure 6.1b).

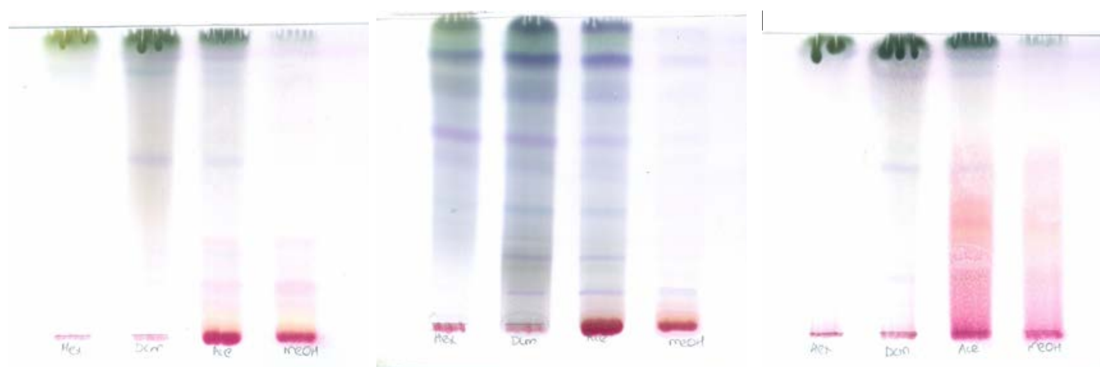


Fig. 1a. Expression on CEF Fig. 1b. Expression on BEA Fig. 1c. Expression on EMW

The TLC plates were shown here at a magnification of ×0.563

Figure 6.1: Expression of fractions of Acetone extracts of *A. korupensis* using three different expression methods.

6.3.4 Cell viability and cytotoxicity assays.

PBMC were confirmed viable since the cell culture media gradually used up the phenol red in the medium and changed the colour from orange to pale yellow over a period of 7 days. The plates inoculated with ASF NIG/99 virus showed distinct rosette formations around the macrophages, an indication that the macrophages were infected and haemadsorbed with the pig red blood cells in the medium. There was no visible reduction in cell population when compared with cells inoculated for diagnostic purposes and no rosette formations were visible in the plates inoculated with placebo (wash buffer only). Complete or partial CPE was observed with concentrations of extract ≥5mg/ml and for the pure extract diluent (DMSO), however a 1:1000 and lower dilutions of the diluent was non cytotoxic to the PBMC; there was no apparent

reduction in the macrophages population and rosette formations developed normally compared to the cells without the diluent.

6.3.5 Antiviral Assay of extract of A. korupensis and its fractions on ASF virus

Cells from PBMC grew normally until approximately 96 hours post-infection following which some reductions in rosette formations were observed. However, after 120 hours, marked reduction in the population of Macrophages and CPE were observed indicating cell deaths. Cell culture plates were read approximately 108 -109 hours post-treatment. Noticeable reductions in the quantity of rosette formations were observed in wells treated with acetone, dichloromethane and methanol extracts and also in wells treated with hexane, methanol and ethyl acetate fractions of acetone extract (Table 6.4). Hexane and chloroform extracts and chloroform fraction of acetone extract showed minimal reduction in the number of rosettes observed and counted, indicating weak activities against ASFV (Table 6.4).

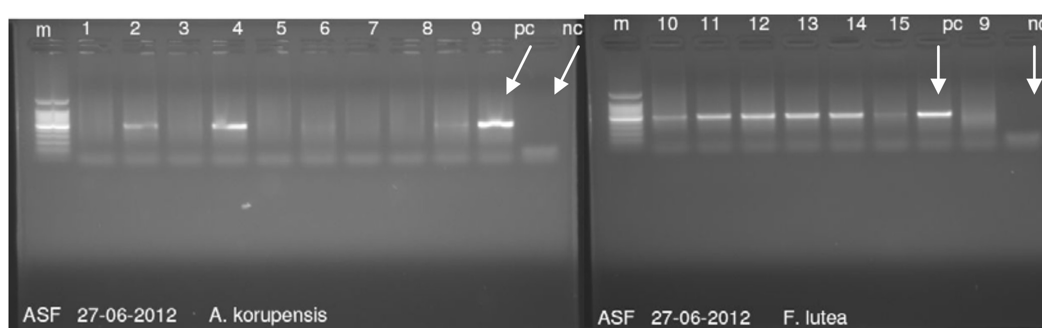
Table 6.4. Observation of activities of *Ancistrocladus korupensis* extracts and fractions on haemadsorbing African swine fever virus *in-vitro* 5-days post infection.

	1	2	3	4	5	6	7	8	9	10	11	12
Mg/ml	ASF NIG/99 (+)	Acetone (C)	Hexane(C)	DCM (C)	Chloroform (C)	Methanol (C)	Hexane (F)	Methanol (F)	Ethyl acetate (F)	Chloroform (F)	Blank	Diluent (-)
1	+++	-	+	-	++	-	-	-	-	+		-
0.5	+++	-	++	-	++	-	-	-	-	+		-
0.25	+++	-	++	-	++	-	-	-	-	++		-
0.125	+++	-	+++	-	+++	-	-	-	+	++		-
0.0625	+++	-	+++	+	+++	-	-	+	+	+++		-
0.03125	+++	+	+++	++	+++	++	+	++	++	+++		-
0.015625	+++	++	+++	++	+++	++	++	++	++	+++		-
0.0078125	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++		-

+++ = ≥50 rosettes were observed and counted in the well; ++ = 10-50 rosettes were observed and counted; + = ≤10 rosettes were observed and counted. - = no rosette was observed. Note: Rosette formation is an indication of virus infection of the macrophage cells in the PBMC medium. Blank contained no diluent, cells or viruses.

6.3.6 PCR and Real-time PCR (qPCR) assays

The PCR results confirmed the observed reduction in rosette formation associated with reduced activities of ASFV *in-vitro* in the presence of *A. korupensis* extracts or fractions. Acetone, Dichloromethane, Methanol extracts and hexane, methanol and ethyl acetate fractions of Acetone extract were effective against the ASFV as no 478bp product was observed (Figure 6.2, Lanes 1, 3, 5-8). However, Hexane extract and Chloroform fraction displayed partial activities while Chloroform extract showed no activity against ASFV. The *Ficus lutea* plant control showed no activity against ASFV (Figure 6.2, Lane 10-15). Both the positive and negative controls passed the internal quality control test required to accept the results (see Figure 6.2)



m=marker; pc=positive control (ASF NIG/99); nc=negative control (wash buffer); AK= Ancistrocladus korupensis; FL= Ficus lutea; Lane 1=Acetone extract(AK); Lane 2=Hexane extract(AK); Lane 3=Dichloromethane extract(AK); Lane 4=Chloroform extract(AK); Lane 5=Methanol extract(AK); Lane 6=Hexane fraction(AK); Lane 7=Methanol fraction(AK); Lane 8=Ethyl acetate fraction(AK); Lane 9=Chloroform fraction(AK); Lane 10=Acetone extract(FL); Lane 11=Water extract (FL); Lane 12=Chloroform extract (FL); Lane 13=Butanol extract(FL); Lane 14=Ethyl acetate extract(FL); Lane 15=Hexane extract(FL)

Figure 6.2. Gel image showing PCR results and the effect of A. korupensis on ASF virus.

Note that harvested samples (1mg/ml dilutions) from the PBMC were used to carry out the PCR assays.

No detectable level of ASF viral genome was observed from the qPCR done since the fluorescent measurement was not above the background signal for any of the tested samples and no sigmoid-shaped curve was observed.

6.3.7 Re-infectivity Assay of extract of A. korupensis

None of the cultures containing plant extracts (acetone, dichloromethane and methanol) and its fractions (hexane, methanol and ethyl acetate fractions) showed rosette formation 96 and 120 hours post-inoculation indicating the lack of infectious virus in the inoculum. However, the positive control wells (ASF NIG/99 virus) displayed characteristic growth patterns and rosette formations that were comparable to the expected standards. No growth was observed in the negative controls (wash buffer). Observed cytotoxicity was also similar to what was previously reported in the cytotoxicity assay.

6.4. DISCUSSION

Certain important plant metabolites were found in abundance in the analysed plant including cardiac glycosides, alkaloids and steroids. Cardiac glycoside has been employed in the treatment of congestive heart failure and cardiac arrhythmia. The virus affects many visceral organs including the heart and it has been suggested previously that death following ASFV infection may be due to heart failure [31]. It is opined that this plant positively influences cardiac outputs by increasing the force of contraction through its effect on the sodium-potassium pumps in the cell membrane [32]. In addition, alkaloids and steroids from this plant should have various pharmacological effects and may minimize the effect of the virus on the pig cells during *in-vivo* infection. Additional research is required to determine the particular metabolites or combinations of metabolites that are responsible for the therapeutic claims ascribed to this plant by the farmers.

Gas chromatography has been known to separate large numbers of compounds in a single analysis and in combination with mass spectrometry (GC-MS) usually results

in a highly selective and sensitive method of chemical compound analysis in plants [33]. In this analysis, we used the method of GC-MS to identify at least 35 compounds from the plant *A. korupensis*. These compounds are consistent with those of previous reports [17,18,20]. The range of activities of these compounds extends from anti-tumour, febrifugal, virucidal, anti-insulin, antibacterial, prostate treatment, vaccine constituents to anti-hypercholesterolemic [34-37]. While activities of this plant against HIV and certain other human viruses have been evaluated [18], this is the first report of its use against animal viruses specifically. Though, the particular compound or interaction of compounds that was responsible for this anti-ASF activity in crude extracts and fractions used in this study are not yet known, the *in-vitro* results support the claim made by farmers of effectiveness of this plant in the management of ASFV. Based on the re-infectivity assay, some extracts and certain fractions of the plant have good virucidal activities which can be positively explored. The cell culture system results were further supported by conventional PCR and QPCR. It is possible that the extractants used in this study possibly affect the effectiveness of the QPCR system since it is expected that QPCR will be more sensitive than the conventional PCR. Further testing of each identified compound for individual and combined ranges of biological activity against ASFV are imperative.

It will also be important to carefully examine the cause of cytotoxicity in this plant and devise ways of eliminating or reducing this effect in view of the substantial therapeutic potential of this plant. While the extracts and fractions significantly reduced ASFV titres, they also did significant damage and caused major reduction in PBMC populations in the culture. Laird and Lisinge [38] and Laird [39] previously reported on the toxicity associated with the *A korupensis* and this effect appeared to be cumulative in this study.

It is possible that there is continuous intra-cytoplasmic absorption of *A. korupensis* by the PBMC and the post-96 hour levels of absorption were incompatible with the survival of the macrophages due to this increasing toxicity [37-39]. Thus, since the toxic dose level appears to be quite close to the anticipated effective antiviral dose against ASFV, the plant likely has a narrow therapeutic index in the field. It is possible that some of the pigs that initially recovered following treatment with *A. korupensis* but later died, may have succumbed to the toxic effect of the plant. While this plant holds potential for the treatment of certain viral infections in pigs, including ASF, its cytotoxicity remains a concern that will require *in-vivo* assessment of acute and chronic toxicity in live animals in order to validate the effectiveness and therapeutic index of *A. korupensis* in the management of ASF in pigs.

The water extraction method poorly expressed the active plant compounds and most of the expressed compounds are basic to neutral. However, since water is an important medium for drug administration, it will be important to conduct additional studies to validate how water may be used in the administration of compounds and extracts from *A. korupensis*.

6.5. CONCLUSION

In conclusion, the farmers' claims of the effectiveness of the use of *Ancistrocladus korupensis* in the management of ASF seem to have a degree of empirical support. Our experiment has provided evidence and confirmed that the extracts and fractions of extract from the plant have antiviral/virucidal activities against ASF virus. It significantly reduced both the viral titres to undetectable level in the molecular biology experiment and terminated the infectiousness of the virus *in-vitro*. However, the cytotoxic effect of the plant will need to be overcome in order to reduce the

negative effect of the plant while still harnessing its therapeutic potential. Further research on the antiviral compounds and effect of the plant holds potential for uncovering a novel antiviral compound and should be explored further for this and other animal viruses for which treatment options are either limited or non-existent.

Competing interests

We do not have any competing financial, business or moral interest that should prevent the publication of this article if accepted.

Authors' contributions

FOF, LH, ADSB conceived the project and draw up the protocols. MMF identified and prepared the plant for laboratory analysis. FOF and OOO carried out the TLC and extraction procedures; OOO+, MMF and FOF carried out the phytochemical screening and GC-MS; FOF did the cell culture and molecular biology works. LH, ADSB and FOF carried out the analyses. All authors contributed to the drafting of the final manuscript and approved it for submission.

Acknowledgement

We wish to thank Ms Dipolelo Semenya and Nontobeko Mtshali & Dr. Juanita van Heerden for assistance with supplies of primary cell cultures and molecular biology respectively; and Ms Selaelo Raphatlelo for the TLC. Members of the ASF Project Implementation Task Team (ASF PITT) of the National Veterinary Research Institute, Vom, Nigeria are thanked for their inputs. Special thanks to Prof. J. N. Eloff for permitting the use of his lab for certain aspects of the work, The Executive Secretary, Agricultural Research Council of Nigeria, Abuja for part funding of this

project under the Project Code 025060410100000 (*Development of rapid and effective Diagnostic and Control tools for African Swine Fever*) and the Executive Director, National Veterinary Research Institute, Dr. M. S. Ahmed for his support.

6.6 REFERENCES

[1]. World Organization for Animal Health (OIE): **Early detection and contingency plans for African swine fever**. *Recommendation 2 of the 24th Conference of the OIE Regional Commission for Europe*, Astana, Kazakhstan, 20-24 September, 2011. Available at: http://www.oie.int/doc/ged/D10785.PDF. Accessed on 11 March, 2012.

[2]. World Organization for Animal Health (OIE): **WAHID Interface: Animal Health Information. Weekly Summary**. Available at: http://web.oie.int/wahis/public.php?page=weekly_report_index. Accessed on 11 March, 2012.

[3]. King K, Chapman D, Argilaguet JM, Fishbourne E, Hutet E, Cariolet R, Hutchings G, Oura CAL, Netherton CL, Moffat K, Taylor G, Le Potier M-F, Dixon LK, Takamatsu H-H: **Protection of European domestic pigs from virulent African isolates of African swine fever by experimental immunisation.** *Vaccine* 2011, **29**:4593-4600.

[4]. Dixon LK, Escribano JM, Martins C, Rock DL, Salas ML, Wilkinson PJ: ***Asfarviridae***, In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA, (eds), *Virus Taxonomy, VIIIth Report of the International Committee on Taxonomy of Viruses*, Elsevier Academic Press, London, 135–143, 2005.

[5]. Sofowora A, *Medicinal Plants and Traditional Medicine in Africa*, John Wiley and Sons Ltd., 150-153, 1993.

[6]. Kudi AC, Myint SH: **Antiviral activity of some Nigerian medicinal plant extracts**. *J Ethnopharmacol* 1999, **68**: 289-294.

[7]. Kudi AC, Umoh JU, Eduvie LO, Gefu J: **Screening of some Nigerian medicinal plants for antibacterial activity**. *J Ethnopharmacol* 1999, **67**: 225-228.

[8]. Akinyemi KO, Oladapo O, Okwara CE, Ibe CC, Fasure KA: **Screening of crude extract of six medicinal plants used in South-West Nigerian unorthodox medicine for anti-methicillin resistant Staphylococcus aureus activity**. *BMC Complement Alternative Med* 2005, **5**:6 doi:10.1186/1472-6882-5-6.

[9]. Odugbemi T: *Medicinal plant database from Nigeria* (Excel format). Available online at http://www.medicinalplantsinnigeria.com/Plantsdatabase.xls. Accessed on 15th December, 2011.

[10]. Vanden Berghe DA, Vlietinck AJ, Vanhoof L: **Plant products as potential antiviral agents**. *Bulletin Institute Pasteur* 1986, **84**: 101-147.

[11]. Ying-Wang C, Huang S-C, Zhang Y, Lai Z.-R, Kung S-H, Chang Y-S, Lin C-W: **Antiviral ability of *Kalanchoe gracilis* leaf extract against Enterovirus 71 and Coxsackievirus A16**. *Evidence-Based Complement Alternative Med* 2012, Article ID **503165**; doi: 10.1155/2012/503165,.

[12]. Silva O, Barbosa S, Diniz A, Valdeira ML, Gomes E: **Plant extracts antiviral activity against Herpes simplex virus type 1 and African swine fever**. *Pharmaceutical Biol* 1997, **35**: 12-16.

[13]. Madureira AM, Ascenso JR, Valdeira L, Duarte A, Frade JP, Freitas G, Ferreira MJU: **Evaluation of the antiviral and antimicrobial activities of Triterpenes isolated from *Euphorbia segetalis***. *Natural Product Res* 2003, **17**: 375-380.

[14]. Galindo I, Hernaez B, Berna J, Fenoll J, Cenis JL, Escribano JM, Alonso C: **Comparative inhibitory activity of the stilbenes resveratrol and oxyresveratrol on African swine fever virus replication**. *Antiviral Res* 2011, **91**: 57-63.

[15]. Thomas DW, Gereau RE: **Ancistrocladus korupensis (Ancistrocladaceae): A new species of Liana from Cameroon**. *Novon* 1993, **3**: 494-498.

[16]. Foster PF, Sork VL: **Population and genetic structure of the West African rain forest Liana *Ancistrocladus korupensis* (Ancistrocladaceae)**. *American J Botany* 1997, **84**: 1078-1091.

[17]. Hallock YF, Manfredi KP, Blunt JW, Cardelinna JH, Schaffer M, Gulden K-P, Bringmann G, Lee AY, Clardy J, Francois G, Boyd MR: **Korupensamine A-D, novel antimalarial alkaloids from Ancistrocladus korupensis**. *J Organic Chem* 1994, **59**: 6349-6355.

[18]. Boyd MR, Hallock YF, Cardelinna JH, Manfredi KP, Blunt JW, McMahon JB, Buckheit Jr RW, Bringmann G, Schaffer M, Cragg GM, Thomas DW, Jato JG: **Anti-HIV Michellamines from *Ancistrocladus korupensis***. *J Medicinal Chem* 1994, **37**: 1740-1745.

[19]. Schwikkard S, van Heerden FR: **Antimalarial activity of plant metabolites**. *Natural Product Rep*, 2002, **19**: 675-692.

[20]. McMahon JB, Currens MJ, Gulakowski RJ, Buckheit Jr. RW, Lackman-Smith C, Hallock YF, Boyd MR: **Michellamine B, a Novel Plant Alkaloid, Inhibits Human Immunodeficiency Virus-Induced Cell Killing by at Least Two Distinct Mechanisms**. *Antimicrobial Agents Chemotherapy* 1995, **39**: 484–488.

[21]. Trease GE, Evans WC: *A Textbook of Pharmacognosy*. 1st Ed., Williams and Wilkins Company, Baltimore, Maryland, 1966.

[22]. Harborne JB: *Phytochemical Methods*. Chapman and Hall, New York, NY, USA, 1973.

[23]. Rahim G, Qureshi R, Gulfraz M, Arshad M, Rahim S: **Preliminary phytochemical screening and ethnomedicinal uses of *Teucrium stocksianum* from Makaland Division**. *J Medicinal Plant Res* 2012, **6**: 704-707.

[24]. Ivanov V, Sandell E: **Characterization of Polychlorinated Biphenyl Isomers in Sovol and Trichlorodiphenyl formulations by high-resolution Gas Chromatography with Electron Capture detection and high-resolution Gas Chromatography-Mass Spectrometry techniques**. *Environmental Science Technol* 1992, **26**: 2012-2017.

[25]. Niessen WMA: *Current Practice of Gas Chromatography-Mass Spectrometry*. Marcel Dekker, CRC Press, New York, 2001.

[26]. National Institute of Standards-USA (NIST): *Standard Reference Data*. Available online at http://www.nist.gov/srd/, Accessed on 15th June, 2010.

[27]. Shimadzu Corporation. *GC-MS QP 2010 Plus brochure*. Available online at http://www.ssi.shimadzu.com/products/literature/Mass_Spec/GCMS-QP2010_Plus_F.pdf, Accessed on 11th June, 2012.

[28]. Kotze M, Eloff JN, **Extraction of antibacterial compounds from *Combretum microphyllum* (Combetaceae)**. *South African J Botany*, 2002, **68**: 62-67.

[29]. Bastos ADS, Penrith M-L, Crucière C, Edrich J, Hutchings G, Roger F, Couacy-Hymann E, Thomson GR: **Genotyping field strains of African swine fever virus by partial *p72* gene characterization**. *Archives Virol,* 2003, **148**: 693–706.

[30]. King DP, Reid SM, Hutchings GF, Grierson SS, Wilkinson PJ, Dixon LK, Bastos ADS, Drew TW: **Development of a TaqMan PCR assay with internal**

amplification control for the detection of African swine fever virus. *J Virol Met* 2003, **107**: 53-61.

[31]. Guo L, Qian JY, Abrams R, Tang HM, Weiser T, Sanders MJ, Kolaja KL: **The electrophysiological effects of cardiac glycosides in human iPSC-derived cardiomyocytes and in guinea pig isolated hearts**. *Cellular Physiol Biochem* 2011, **27**: 453-462.

[32]. Choi YH, Kim HK, Verpoort R: **Metabolomics**, In: Kayser O, Quax WJ, (eds.), *Medicinal Plant Biotechnology: From Basic Research to Industrial Application* (Volume 1), Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim, Germany, pp 9-28. Available online at: http://onlinelibrary.wiley.com/book/10.1002/9783527619771, 2007.

[33]. Wentland MP, Bailey DM, Cornett JB, Dobson RA, Powles RG, Wagner RB: **Novel amino-substituted 3-quinolinecarboxylic acid antibacterial agents: synthesis and structure-activity relationship**. *J Medicinal Chem* 1984, **27**: 1103-1108.

[34]. Dexter DL, Hesson DP, Ardecky RJ, Rao GV, Tippett DL, Dusak BA, Paull KD, Plowman J, DeLarco BM, Narayanan VL, Forbes M: **Activity of a novel 4-quinolinecarbocylic acid, NSC 368390 [6-fluoro-2-(2′ fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt] against experimental tumours**. *Cancer Res* 1985, **45**: 5563-5568.

[35]. Bentley KW: ***β*-Phenylethylamines and the isoquinoline alkaloids**. *Natural Product Rep* 1996, **13**: 127-150.

[36]. Kelly GS: **Squalene and its potential clinical uses**. *Alternative Med Rev* 1999, **4**: 29-36.

[37]. Cragg GM, Newman DJ: **Drug discovery and development from natural products: The way forward**. *Proceedings of the 11th Natural Product Research for Eastern and Central Africa (NAPRECA) Symposium,* 9-12 August 2005, Hôtel Panorama, Antananarivo, Madagascar, 2005, **11**: 56-69.

[38]. Laird S, Lisinge E: **Case studies from Cameroon: Contrasting benefit-sharing in the pharmaceutical and phytomedical industries**. Case studies submitted to the CBD Secretariat, UNEP/CBD/COP/4/Inf.25, – 49 pp, Available online at http://www.biodiv.org/doc/case-studies/cs-abs-aristo.pdf, 1998, Accessed on 21st June, 2012.

[39]. Laird SA: **The Convention on Biological Diversity: changing ethical and legal frameworks for biodiversity research and prospecting**. *Unasylva* 2003-***206*** *- Global Conventions Related to Forests*, **52**: 2001/3, Available online at: http://www.fao.org/docrep/003/y1237e/y1237e00.htm#TopOfPage, , Accessed on 21st June, 2012.

7.0 LENGTH AND SEQUENCE HETEROGENEITY OF THE *ASFIVIRUS THYMIDINE KINASE* GENE

FOLORUNSO O. FASINA[1, 2], JENNIFER L. EDRICH[1, 3], LIVIO HEATH[4], CARMINA GALLARDO[5], WILNA VOSLOO[4#] & ARMANDA D. S. BASTOS[1,*]

[1] *Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa.*

[2] *Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort 0110, South Africa.*

[3] *Department of Biomedical Sciences, Tshwane University of Technology, Pretoria, South Africa*

[4] *Transboundary Animal Disease Programme, ARC-Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort 0110, South Africa.*

[5] *Community Reference Laboratory for African swine fever, CISA-INIA, Valdeolmos, 28130, Madrid, Spain.*

Present address: Australian Animal Health Laboratory, 5, Portalington Road, East Geelong VIC 3219, Australia.

*Corresponding author: Prof. A.D.S. Bastos

Tel: +27 12 420 4612. Fax: +27 12 362 5242

E-mail: ADBastos@zoology.up.ac.za

Aim: *To compare and contrast the TK gene of African swine fever virus field strains in relation to genotypic and pathogenic variations.*

7.0.1 Abstract

The *thymidine kinase* (*TK*) gene of African swine fever (ASF) viruses representative of 21 of the 22 presently recognised *p72* genotypes was amplified and sequenced with primers binding in regions flanking this gene. Sequence analyses revealed that the *p72* gene and *TK* gene phylogenies recovered the same three major evolutionary lineages, whilst translation of the nucleotide sequences revealed that the presence of viruses containing frameshift mutations result in premature stop codons. Truncated proteins of either 185 (East African isolates) or 188/189 (southern African isolates) amino acids were predicted to result instead of the expected full-length 196 amino acid (aa) enzyme. In addition, these stop codons were generally followed by nonsense insertions of varying lengths, resulting in a larger than expected amplification product. A possible link between truncated *TK* gene products and the sylvatic cycle is suggested.

Running title: *Asfivirus thymidine kinase* heterogeneity

Key words: African swine fever virus, thymidine kinase, frameshift mutation, truncated protein, phylogeny.

7.1 Introduction

African swine fever virus (ASFV) is the only member of the family *Asfarviridae* [1]. It causes a highly infectious disease in domestic pigs with mortality rates of up to 100 % in extremely pathogenic strains [2] and recovered swine may become carriers of the disease [3]. In southern and East Africa, ASFV is maintained in an ancient sylvatic cycle between warthogs and ticks of the *Ornithodoros moubata* complex [4, 5], and this cycle have a link with the heterogeneity of the virus in East Africa [5]. With respect to West Africa, although the distribution of warthogs ranges throughout the savannah belt from Cameroon to Senegal [6], *Ornithodoros* species have only been detected in Senegal [7]. Thus, there is no evidence that the sylvatic cycle occurs in West Africa and maintenance and transmission of the infection in this region occurs without any apparent involvement of either sylvatic suids or ticks [4].

During replication of ASFV in the cytoplasm of host cells, the virus encodes enzymes involved in transcription and DNA synthesis [8], including four enzymes involved in the synthesis of deoxynucleotide precursors, namely thymidine kinase (TK) [9-11], thymidylate kinase [12], ribonucleotide reductase (RR) [13], and deoxyuridine triphosphatase [14].

The *TK* gene of ASFV is a single-copy gene situated within the ORF *K196R* [8]. The gene appears to have highly conserved nucleotide binding motifs [10] and codes for a 196 amino acid polypeptide [11]. ASFV-infected cells show increased RR [13] and TK activities [15], corresponding to the synthesis of viral proteins within the infected host cell [9]. It has been suggested that these enzymes could be involved in overcoming the allosteric regulation of the corresponding cellular enzymes, or in targeting the enzymatic activity to the most suitable subcellular site for viral infection [8].

Following insertional inactivation of TK, the virulence of vaccinia virus decreases [16], as does the virulence of ectromelia virus [17], herpes simplex virus [18] and marmoset herpesvirus [19]. In 1998, Moore and co-workers created *TK* deficient (*TK*$^{-}$) mutants of ASFV [15]. Subsequent sequencing analysis revealed two types of mutants, M1 and M2, having an adenosine deletion in codon 40 and 41 respectively, resulting in a frameshift mutation and subsequent translation termination at codons 41 and 71 respectively. Infection of domestic swine with a *TK*$^{-}$ strain showed reduced virulence [15], and swine that recovered from the infection were protected from developing ASF from the parental pathogenic strains. These findings suggest that the *TK* gene is not essential for virus replication but does affect virulence [15]. It is known that ASF field strains can vary markedly with respect to pathogenicity [20]. This study aimed to assess heterogeneity of the *TK* gene of ASF field strains in relation to viral virulence, epidemiological cycle and evolutionary lineage assignment.

7.2 Materials and methods

7.2.1 *Selection of candidate viruses*

The candidate viruses (103 isolates) used in this study were selected from the repository of the ASF viruses available at the Transboundary Animal Disease Programme (TADP) ARC-Onderstepoort Veterinary Institute. A further 16 isolates were obtained from the European Union Community Reference laboratory for ASF (CISA-INIA, Valdeolmos, Spain) virus repository (Table 7.1). These viruses were previously classified based on pathogenicity as highly virulent, moderately virulent or of low virulence and were also haemadsorbing or non-haemadsorbing (not indicated in this work). All viruses were *p72* genotyped as described previously [1], with the genotype of many having been reported in previous studies (summarised in Table

7.1). A total of 119 isolates including certain isolates from Nigeria banked at CISA-INIA and those representative of known genotypes occurring throughout sub-Saharan Africa and from areas where the virus has made historical incursions were included in this study (Table 1).

7.2.2 *DNA extraction, amplification and purification*

DNA was extracted from cell cultured viruses using a modified silica/guanidium-based method [21]. The majority of the viruses were genotype I viruses representatives of the major ESAC-WA *p72*-PCR lineages (Table 1) [1, 5, 22], which were also confirmed by the CVR analyses.

A 759 bp segment, corresponding to the entire *TK* gene and flanking regions, was targeted using primers TK1 (5' CGC GTC TTA CTA AAA GTG A 3') and TK-Rev (5' TAG CAG AGT AAT AAA CTC TT 3') designed specifically for this study on the basis of the guidelines of Rychlik [23]. Final concentrations in a 50 µl PCR reaction mix were 0.2 mM dNTP, 75 mM Tris HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 20 mM (NH_4) and 0.4 µM of each primer in the presence of 1U thermostable Taq DNA polymerase (Biotools, Madrid, Spain). The thermal cycling profile consisted of an initial cycle of 96 °C for 20 seconds, 48 °C for 30 seconds and 70 °C for 45 seconds, followed by 34 cycles of 96 °C for 12 seconds, 47 °C for 20 seconds and 70 °C for 40 seconds.

The amplified products were run on a 1.5% agarose gel containing Goldview nucleic acid stain against a 100 bp ladder (Promega, Madison, USA).

The *TK*-PCR products were purified using the High Pure PCR Product Purification kit (Roche) and cycle sequencing was carried using the PCR amplification primers (TK1

and TK-Rev). The sequencing reactions were performed according to the manufacturer's specifications using the Big Dye Terminator v. 3.1 Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, USA). Cycling conditions were 25 cycles of: 96 °C for 10 seconds, annealing at 48°C for 5 seconds and 60 °C for 4 minutes. The DNA was precipitated using a sodium acetate method and samples were run on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA).

7.2.3 *Sequence analyses*

Sequence chromatograms were edited and aligned with the Chromas program in the MEGA (Version 5.03) [24]. Distance trees were generated using and the neighbor-joining algorithm in MEGA 5.03 [24] and maximum parsimony trees were inferred using PAUP* Version 4.0b10 [25]. To determine the degree of support for each node, data were resampled between 1000 and 10 000 times using the bootstrap method and branches with less than 70 % support were collapsed [26]. The best-fit model selected under the AIC in Mega 5.03, the Tamura-Nei distance correction method was used to infer distance trees [27]. Maximum parsimony employed the heuristic search method with gaps inserted for alignment purposes being treated as a 21st character state in PAUP* [25].

7.3 Results

It was observed that all *TK*-PCR products were of the same size with the exception of 30 viruses of predominantly East African origin, including: [189aa in BUR 84/2; 188aa in MAL 1978, LIL 20/1, MAL2002/1, MKUZI GR 21-23, MKUZI GR 21-11, MKUZI GR 22-6, KAC 91/2, RSA P/1/95, ZAW 88/1; and 185aa in KEN 1950, MZI 92/1, SUM 14/11, NYA 1/2AB, TAN 1/01, TAN 2003/1, UGA 1/95, UGA 95/3,

UGA 2003/1, VIC UGA, KEN 05 Tk1, KEN 06 Bus, BUR 84/1, KEN 01/2, KEN 01/6, KEN 01/5, KEN 01/3, KEN 01/1, Hinde II and KWH 12], which all produced bands larger than the expected size. The size of the coding ORF for the TK protein also varied between isolates (Table 7.1, Appendix E).

The majority of isolates encoded a protein of 196 amino acids. These isolates comprised of viruses from Europe, West, Central and Southern Africa. A smaller *TK* product of 185 amino acids, arising from a frameshift mutation at position 561 and resulting in a stop codon immediately thereafter, was observed in KEN 1950, MZI 92/1, SUM 14/11, NYA 1/2AB, TAN 1/01, TAN 2003/1, UGA 1/95, UGA 95/3, UGA 2003/1, VIC UGA, KEN 05 Tk1, KEN 06 Bus, BUR 84/1, KEN 01/2, KEN 01/6, KEN 01/5, KEN 01/3, KEN 01/1, Hinde II and KWH 12.

MAL 1978, LIL 20/1, MAL2002/1, MKUZI GR 21-23, MKUZI GR 21-11, MKUZI GR 22-6, KAC 91/2, RSA P/1/95, ZAW 88/1 also had a frameshift mutation at position 561, resulting in a stop codon at position 571, and an encoding a *TK* product of 188 amino acids. These isolates having smaller *TK* gene products of 185 amino acids were all East African ASFV strains. BUR 84/2 however has a *TK* gene product of 189 amino acids. Despite the smaller TK protein product size, nucleotide sequence alignment revealed that the considerably larger *TK*-PCR products which were visually observed as described above were due to nonsense insertions of differing lengths at position 596 (Table 7.1).

The figures 7.1 and 7.2 showed the phylogenetic relationship of the viruses used in this study using mid-point rooted and unrooted neighbour-joining trees which depict the Thymidine kinase gene relationship of ASF viruses. These results were consistent with the previous classifications of the viruses using the *p72* and CVR genetic studies.

TABLE 7. 1. Summary of the viruses characterised in this study

Isolate	Country of origin	Year of Outbreak	Species origin	*p72* genotype	Source of sequence used to infer genotype	Predicted TK protein size	pI / MW*	Genbank Accession No	Pathogenicity	Reference
1. LIS 57	Portugal	1957	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6	AF504895*	H	This study
2. Dakar/59	Senegal	1959	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6		H	This study
3. *LIS 60	Portugal	1960	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6	AF504891	H	This study
4. Angola/70	Angola	1970	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6			This study
5. E70(MS44)	Spain	1970	*Sus scrofa*	I	Zsak et al. 2005	196aa	9.37 / 22420.6	M63119		Hernandez & Tabares 1991
6. BA71(V)	Spain	1971	*Sus scrofa*	I	Yanez et al. 1995	196aa	9.37 / 22420.6	Z21490 U18466		Blasco et al. 1990 Yanez et al. 1995
7. *Malta 78	Malta	1978	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6	AF504892	H	This study
8. NAM/1/80	Namibia	1980	*Phacochoerus africanus*	I	Bastos et al. 2003	196aa	9.37 / 22420.6			This study
9. LIV/5/40	Zambia	1982	*Ornithodoros*	I	Lubisi et al. 2005	196aa	9.37 / 22420.6			This study
10. *CAM 4/85	Cameroon	1985	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6	AF504896		This study
11. CAM/1/86	Cameroon	1986	*Sus scrofa*	I	This study	196aa	9.37 / 22420.6			This study
12. DRC624/89	DRC	1985	*Sus scrofa*	I	This study	196aa	9.44 / 22438.6			This study
13. SPEC 208	Namibia	1989	*Sus scrofa*	I	This study	196aa	9.37 / 22420.6	AF504899#		This study
14. SPEC 209	Namibia	1989	*Sus scrofa*	I	Boshoff et al. 2007	196aa	9.37 / 22420.6			This study
15. *NUR 1/90	Sardinia	1990	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6	AF504890		This study
16. VICT 90/1	Zimbabwe	1990	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6	AF504902*		This study
17. IC/2/96	Côte d'Ivoire	1996	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6			This study
18. IC/3/96	Côte d'Ivoire	1996	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6			This study
19. NIG 1/99	Nigeria	1999	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6	AF504893		This study
20. MAD 1/98	Madagascar	1998	*Sus scrofa*	II	Bastos et al. 2003	196aa	9.31 / 22393.5	AF504897		This study
21. BOT/1/99	Botswana	1999	*Sus scrofa*	III	Bastos et al. 2003	196aa	9.44 / 22438.6			This study
22. SPEC140	South Africa	1987	*Ornithodoros*	III	This study	196aa	9.44 / 22438.6			This study
23. RSA/W/1/99	South Africa	1999	*Phacochoerus africanus*	IV	Bastos et al. 2003	196aa	9.37 / 22438.6	AF504901		This study
24. MOZ/1960	Mozambique	1960	*Sus scrofa*	V	Bastos et al. 2004	196aa				This study
25. MOZ 94/1	Mozambique	1994	*Sus scrofa*	VI	Bastos et al. 2003	196aa	9.44 / 22390.6	AF504900		This study
26. ZIM/92/1	Zimbabwe	1992	*Sus scrofa*	XVII	Boshoff et al. 2007	196aa	9.31 / 22369.5			This study
27. Spec 120	South Africa	1987	*Sus scrofa*	XIX	Boshoff et al. 2007	196aa	9.44 / 22438.6	AF504898		This study
28. Spec 125	South Africa	1987	*Sus scrofa*	XIX	Boshoff et al. 2007	196aa	9.44 / 22438.6			This study
29. RSA/2/96	South Africa	1996	*Sus scrofa*	XIX	Boshoff et al. 2007	196aa	9.46 / 22478.7			This study
30. RSA/P/3/96	South Africa	1996	*Sus scrofa*	XIX	Boshoff et al. 2007	196aa	9.46 / 22478.7	AF504894*		This study
31. Lillie	South Africa	1973	*Sus scrofa*	XX	Boshoff et al. 2007	196aa	9.46 / 22478.7			This study
32. RSA/1/96	South Africa	1996	*Sus scrofa*	XXI	Boshoff et al. 2007	196aa	9.44 / 22457.7			This study
33. SPEC/254	South Africa	1992	*Sus scrofa*	XXII	Boshoff et al. 2007	196aa	9.37 / 22420.6			This study
34. Rho 61/1	Zimbabwe	1961	*Sus scrofa*	VIII	Bastos et al. 2003	196aa	9.18 / 22359.5			This study
35. Malawi 1978	Malawi	1978	*Sus scrofa*	VIII	Bastos et al. 2003	188aa	9.08 / 21483.4	AF504903*		This study
36. Lil 20/1	Malawi	1983	*Ornithodoros porcinus*	VIII	AY261361	188aa	9.08 / 21483.4	AY261361		Kutish & Rock unpublished
37. MOZ A-98	Mozambique	1998	*Sus scrofa*	VIII	Bastos et al. 2003	196aa	9.18 / 22359.5			This study
38. KAB/6/2	Zambia	1983	*Ornithodoros porcinus*	XI	Lubisi et al. 2005	196aa	9.36 / 22432.8			This study
39. MZI/92/1	Malawi	1992	*Sus scrofa*	XII	Lubisi et al. 2005	185aa	9.18 / 21059.9			This study
40. SUM/14/11	Zambia	1983	*Ornithodoros porcinus*	XIII	Lubisi et al. 2005	185aa	9.33 / 21038.0			This study
41. NYA/1/2	Zambia	1986	*Ornithodoros porcinus*	XIV	Lubisi et al. 2005	185aa	9.49 / 21069.1			This study
42. TAN/1/01	Tanzania	2001	*Sus scrofa*	XV	Lubisi et al. 2005	185aa	9.44 / 21056.0			This study

Isolate	Country of origin	Year of Outbreak	Species origin	*p72* genotype	Source of sequence used to infer genotype	Predicted TK protein size	pI / MW*	Genbank Accession No	Pathogenicity	Reference
43. TAN/2003/1	Tanzania	2003	*Sus scrofa*	XVI	Lubisi et al. 2005	185aa	9.44 / 21056.0			This study
44. UGA/1/95	Uganda	1995	*Sus scrofa*	IX	Bastos et al. 2004	185aa	9.45 / 21070.9			This study
45. Kenya 1950	Kenya	1950	*Sus scrofa*	X	AY261360	185aa	9.45 / 21068.9	AY261360		Kutish & Rock, unpublished
46. Hinde II	Kenya	1959	*Sus scrofa*	X	Bastos et al. 2003	185aa	9.45 / 21098.9	AF504905		This study
47. Kwh/12	Tanzania	1968	***Phacochoerus africanus***	X	Bastos et al. 2003	185aa	9.45 / 21068.9	AF504904		This study
48. BUR 84/1	Burundi	1984	*Sus scrofa*	X	Bastos et al. 2003	185aa	9.45 / 21068.9	AF504906		This study
49. UGA 95/3	Uganda	1995	*Sus scrofa*	X	Bastos et al. 2003	185aa	9.45 / 21068.9	AF504907		This study
50. MOZ/60	Mozambique	1960	*Sus scrofa*	V	Bastos et al., 2004	196aa	9.44 / 22390.5	AF270708	H	This study
51. Mad/62	Madagascar	1962	*Sus scrofa*	I	Bastos et al. 2003	196aa	9.37 / 22420.6	AF449461	H	This study
52. 24823		1977			DQ250110	196aa	9.44 / 22448.6	DQ250110	H	This study
53. Zaire	Zaire	1977	*Sus scrofa*		Gonzague et al., 2001	196aa	9.37 / 22420.6	AY351515	L	This study
54. Mkuzi GR21-23	South Africa	1978	*Ornithodoros porcinus*	XX	Arnot et al., 2009	188aa	9.37 / 21531.5		H	This study
55. Mkuzi GR21-11	South Africa	1978	*Ornithodoros porcinus*	XX	Arnot et al., 2009	188aa	9.37 / 21531.5		H	This study
56. Mkuzi GR22- 6	South Africa	1978	*Ornithodoros porcinus*	XX	Arnot et al., 2009	188aa	9.37 / 21531.5		H	This study
57. MALTA/78	Malta	1978	*Sus scrofa*	I	Bastos et al., 2003	196aa	9.37 / 22420.6	AF301543	H	This study
58. Dominican Republic	Dom. Republic	1979	Cell culture	I	Bastos et al., 2003	196aa	9.37 / 22420.6	AF302810	M	This study
59. Malta	Malta	1979	Cell culture	I	AF301543	196aa	9.37 / 22420.6	AF301543	H	This study
60. SWA Baby warthog 19	Namibia	1980	*Phacochoerus africanus*		AF504881	196aa	9.37 / 22420.6	AF504881		
61. CAM/82	Cameroun	1982	*Sus scrofa*	I	Bastos et al., 2003	196aa	9.37 / 22420.6	AF301544	H	This study
62. BUR 84/2	Burundi	1984	*Sus scrofa*	X	Bastos et al., 2003	189aa	9.51 / 21571.5	AF449464	H	This study
63. Spec 89	Namibia	1986	*Sus scrofa*		Unpublished data	196aa	9.37 / 22420.6			This study
64. HOL/86	Holland	1986	*Sus scrofa*	I	Bastos et al., 2003	196aa	9.37 / 22420.6	AF449467	H	This study
65. ZAW 88/1	Zambia	1988	*Sus scrofa*	VIII	AY351559	188aa	9.08 / 21483.4	AY351559	H	This study
66. OURT 88/2	Portugal	1988	Ornithodoros (*Sus scrofa*)		Unpublished data	196aa	9.37 / 22420.6		H	This study
67. OURT 88/1	Portugal	1988	Ornithodoros (*Sus scrofa*)	I	Bastos et al., 2003	196aa	9.37 / 22420.6	AF3012811	H	This study
68. CAM 89/1	Cameroun	1989	*Phacochoerus africanus*	I	AF511452	196aa	9.37 / 22420.6	AF511452	H	This study
69. Spec 205	Namibia	1989	*Sus scrofa*	I	Arnot et al., 2009	196aa	9.37 / 22420.6	DQ250114		This study
70. KAC 91/2	Malawi	1991	*Sus scrofa*	VIII	Lubisi et al., 2005	188aa	9.46 / 22421.6	AY351504		This study
71. Spec 245	South Africa	1992	*Sus scrofa*	XXII	Boshoff et al., 2007	196aa	9.37 / 22420.6	DQ250117		This study
72. MOZ 94/8	Mozambique	1994	*Sus scrofa*	VI	Bastos et al., 2004	196aa	9.44 / 22390.5	AF270712	H	This study
73. Spec 265	Mozambique	1994	*Sus scrofa*	VI	Bastos et al., 2003	196aa	9.44 / 22390.5	AF270710		This study
74. RSA/P/4/95/+	South Africa	1995	*Sus scrofa*	VII	Unpublished data	196aa	9.46 / 22451.6		H	This study
75. RSA/P/1/95/+	South Africa	1995	*Sus scrofa*	XX	Boshoff et al., 2007	188aa	9.30 / 21503.5	DQ250123	H	This study
76. BEN 97/5	Benin	1997	*Sus scrofa*	I	JX403652	196aa	9.37 / 22420.6	JX403652	H	This study
77. BEN 97	Benin	1997	*Sus scrofa*	I	AF302816	196aa	9.37 / 22420.6	AF302816	H	This study
78. BEN 97/2	Benin	1997	*Sus scrofa*	I	JX403650	196aa	9.37 / 22420.6	JX403650	H	This study
79. BEN 97/3	Benin	1997	*Sus scrofa*	I	JX403651	196aa	9.37 / 22420.6	JX403651	H	This study
80. Benin/P/1/97/+	Benin	1997	*Sus scrofa*	I	Bastos et al., 2003	196aa	9.37 / 22420.6	AF302816	H	This study
81. BEN 97/4	Benin	1997	*Sus scrofa*	I	AY972164	196aa	9.37 / 22420.6	AY972164	H	This study
82. NIG/P/1/98/+	Nigeria	1998	*Sus scrofa*	I	Bastos et al., 2003	196aa	9.37 / 22420.6	AF302817	H	This study
83. NIG/P/2/98/+	Nigeria	1998	*Sus scrofa*	I	Phologane et al., 2005	196aa	9.37 / 22420.6	AY972161	H	This study
84. NIG/P/3/98/+	Nigeria	1998	*Sus scrofa*	I	Phologane et al., 2005	196aa	9.37 / 22420.6	AY972162	H	This study
85. RSA/W/1/99/+	South Africa	1999	*Phacochoerus africanus*	IV	Bastos et al, 2003	196aa	9.37 / 22438.6	AF449477	H	This study
86. NIG/P/1/99/+	Nigeria	1999	*Sus scrofa*	I	Bastos et al., 2003	196aa	9.37 / 22420.6	AF504887	H	This study
87. Bot/P/1/99/+	Botswana	1999	*Sus scrofa*	III	Bastos et al., 2003	196aa	9.44 / 22438.6	AF504886	H	This study

Isolate	Country of origin	Year of Outbreak	Species origin	*p72* genotype	Source of sequence used to infer genotype	Predicted TK protein size	pI / MW*	Genbank Accession No	Pathogenicity	Reference
88. GAM/1/00	Gambia	2000	*Sus scrofa*	I	Bastos et al., 2003	196aa	9.37 / 22420.6	AF449478	H	This study
89. GHA/P/1/00/+	Ghana	2000	*Sus scrofa*	I	Bastos et al., 2003	196aa	9.37 / 22420.6	AF504888	H	This study
90. KEN/2001/2	Kenya	2001	*Sus scrofa*		JX524215	185aa	9.45 / 21056.9	JX524215	H	This study
91. KEN/2001/6	Kenya	2001	*Sus scrofa*		JX403676	185aa	9.45 / 21056.9	JX403676	H	This study
92. KEN/2001/5	Kenya	2001	*Sus scrofa*	X	JX403675	185aa	9.45 / 21056.9	JX403675	H	This study
93. TAN/2001/3	Tanzania	2001	*Sus scrofa*	XV	JX467637	185aa	9.44 / 21056.0	JX467637	H	This study
94. KEN/2001/1	Kenya	2001	*Sus scrofa*	IX	JX403672	185aa	9.45 / 21056.9	JX403672	H	This study
95. RSA/2001/2	South Africa	2001	*Sus scrofa*	VII	JX403671	196aa	9.44 / 22438.6	JX403671	H	This study
96. Cam 2002/4	Cameroun	2002	*Sus scrofa*	I	JX403669	196aa	9.37 / 22420.6	JX403669	H	This study
97. RSA 2002/1	South Africa	2002	*Sus scrofa*		Unpublished data	196aa	9.44 / 22411.5		H	This study
98. Cam 2002/1	Cameroun	2002	*Sus scrofa*	I	JX403666	196aa	9.37 / 22420.6	JX403666	H	This study
99. Cam 2002/3	Cameroun	2002	*Sus scrofa*	I	JX403668	196aa	9.37 / 22420.6	JX403668	H	This study
100. GHA 2002/1	Ghana	2002	*Sus scrofa*	I	JX403662	196aa	9.37 / 22420.6	JX403662	H	This study
101. Cam 2002/5	Cameroun	2002	*Sus scrofa*	I	JX403656	196aa	9.37 / 22420.6	JX403656	H	This study
102. GHA 2002/2	Ghana	2002	*Sus scrofa*	I	JX403663	196aa	9.37 / 22420.6	JX403663	H	This study
103. MAL 2002/1	Malawi	2002	*Sus scrofa*	VIII	AY494553	188aa	9.25 / 21525.5	AY494553	H	This study
104. Cam 2002/2	Cameroun	2002	*Sus scrofa*	I	JX403667	196aa	9.37 / 22420.6	JX403667	H	This study
105. UGA 2003/1	Uganda	2003	*Sus scrofa*	IX	AY351564	185aa	9.45 / 21070.9	AY351564	H	This study
106. Burkina Faso 2003/1	Burkina Faso	2003	*Sus scrofa*	I	JX403661	196aa	9.37 / 22420.6	JX403661	H	This study
107. NAM 2004/3	Namibia	2004	*Sus scrofa*	I	JX403639	196aa	9.37 / 22420.6	JX403639	H	This study
108. NAM 2004/1	Namibia	2004	*Sus scrofa*	I	JX403637	196aa	9.37 / 22420.6	JX403637	H	This study
109. RSA2004/3	South Africa	2004	*Sus scrofa*	IV	JX403645	196aa	9.44 / 22448.6	JX403645	H	This study
110. Kenya05/ Tk1	Kenya	2005	*Sus scrofa*	X	Gallardo et al., 2011	185aa	9.45 /21068.9		H	This study
111. NIG/01/1	Nigeria	2005	*Sus scrofa*	I	JX403677	196aa	9.37 / 22420.6	JX403677	H	This study
112. IC/2/96	Ivory Coast	2005	*Sus scrofa*	I	Bastos et al., 2003	196aa	9.37 / 22420.6	AF302815	H	This study
113. Kenya06 Bus	Kenya	2006	*Sus scrofa*	IX	Gallardo et al., 2009	185aa	9.45 / 2107029		H	This study
114. 1546C/06	Cameroun	2006	*Sus scrofa*		Unpublished data	196aa	9.37 / 22420.6		H	This study
115. Nig 06/PLJs16	Nigeria	2006	*Sus scrofa*	I	KC112582	196aa	9.37 / 22420.6	KC112582	M	This study
116. Nig 06/PlJs42	Nigeria	2006	*Sus scrofa*	I	KC112583	196aa	9.37 / 22420.6	KC112583	L	This study
117. Nig 06/PLJs43	Nigeria	2006	*Sus scrofa*	I	KC112584	196aa	9.37 / 22420.6	KC112584	M	This study
118. Burkina Faso 2007/1	Burkina Faso	2007	*Sus scrofa*	I	JX310054	196aa	9.37 / 22420.6	JX310054	H	This study
119. Mauritius 2007/1	Mauritius	2007	*Sus scrofa*	II	FJ528594	196aa	9.31 / 22393.5	FJ528594	H	This study
120. Nig 08/BNGb2	Nigeria	2008	*Sus scrofa*	I	KC112585	196aa	9.37 / 22420.6	KC112585	M	This study
121. Nig 08/BNGb4	Nigeria	2008	*Sus scrofa*	I	KC112586	196aa	9.37 / 22420.6	KC112586	L	This study
122. Nig 08/BNGb9	Nigeria	2008	*Sus scrofa*	I	KC112587	196aa	9.37 / 22420.6	KC112587	H	This study
123. Nig 08/BNGb24	Nigeria	2008	*Sus scrofa*	I	KC112588	196aa	9.37 / 22420.6	KC112588	L	This study
124. Nig 08/BNMk42	Nigeria	2008	*Sus scrofa*	I	KC112589	196aa	9.37 / 22420.6	KC112589	H	This study
125. Nig 08/LAOk1	Nigeria	2008	*Sus scrofa*	I	KC112590	196aa	9.44 / 22448.6	KC112590	L	This study
126. Nig 08/LAOk2	Nigeria	2008	*Sus scrofa*	I	KC112591	196aa	9.37 / 22420.6	KC112591	M	This study
127. Nig 08/NW6	Nigeria	2008	*Sus scrofa*	I	KC112592	196aa	9.37 / 22420.6	KC112592	L	This study
128. Nig 08/NW10	Nigeria	2008	*Sus scrofa*	I	KC112593	196aa	9.37 / 22420.6	KC112593	L	This study
129. Nig 08/NW12	Nigeria	2008	*Sus scrofa*	I	KC112594	196aa	9.37 / 22420.6	KC112594	L	This study
130. Vic/UGA	Uganda	?	*Sus scrofa*		Unpublished data	185aa	9.45 / 21068.9	-		This study

All unpublished data originated from the laboratories of TADP, ARC-Onderstepoort Veterinary Institute, Onderstepoort, South Africa and the EU Community Reference Laboratory, Valdeolmos, Spain. All genotypes have been confirmed and or submitted as reported in the Table. pI/MW (Isoelectric point per molecular weight) were calculated using ProtParam (Gasteiger et al., 2005).

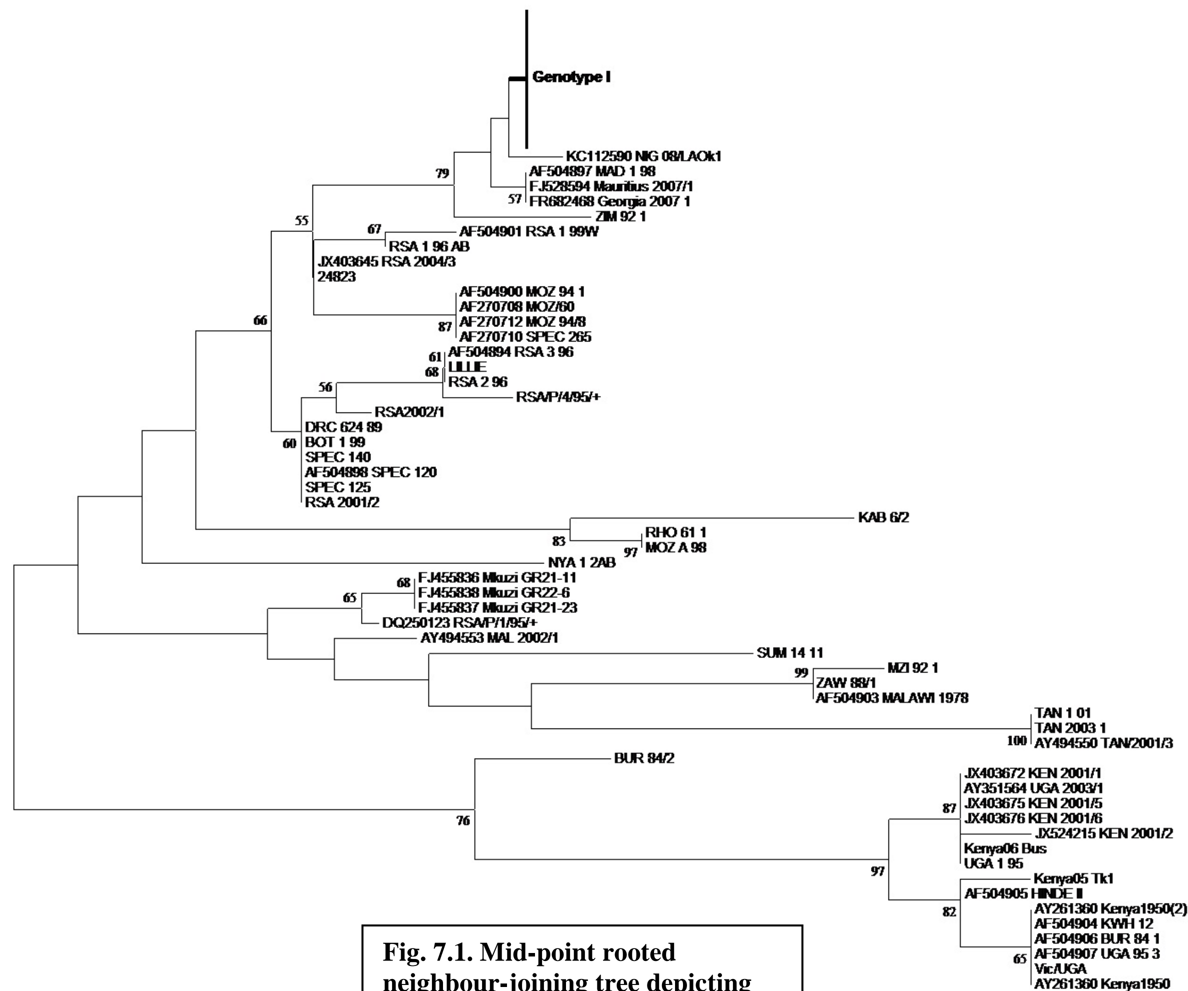


Fig. 7.1. Mid-point rooted neighbour-joining tree depicting the Thymidine kinase gene relationship of ASF viruses. Only those bootstrap values >50% obtained following 10000 replications are indicated.

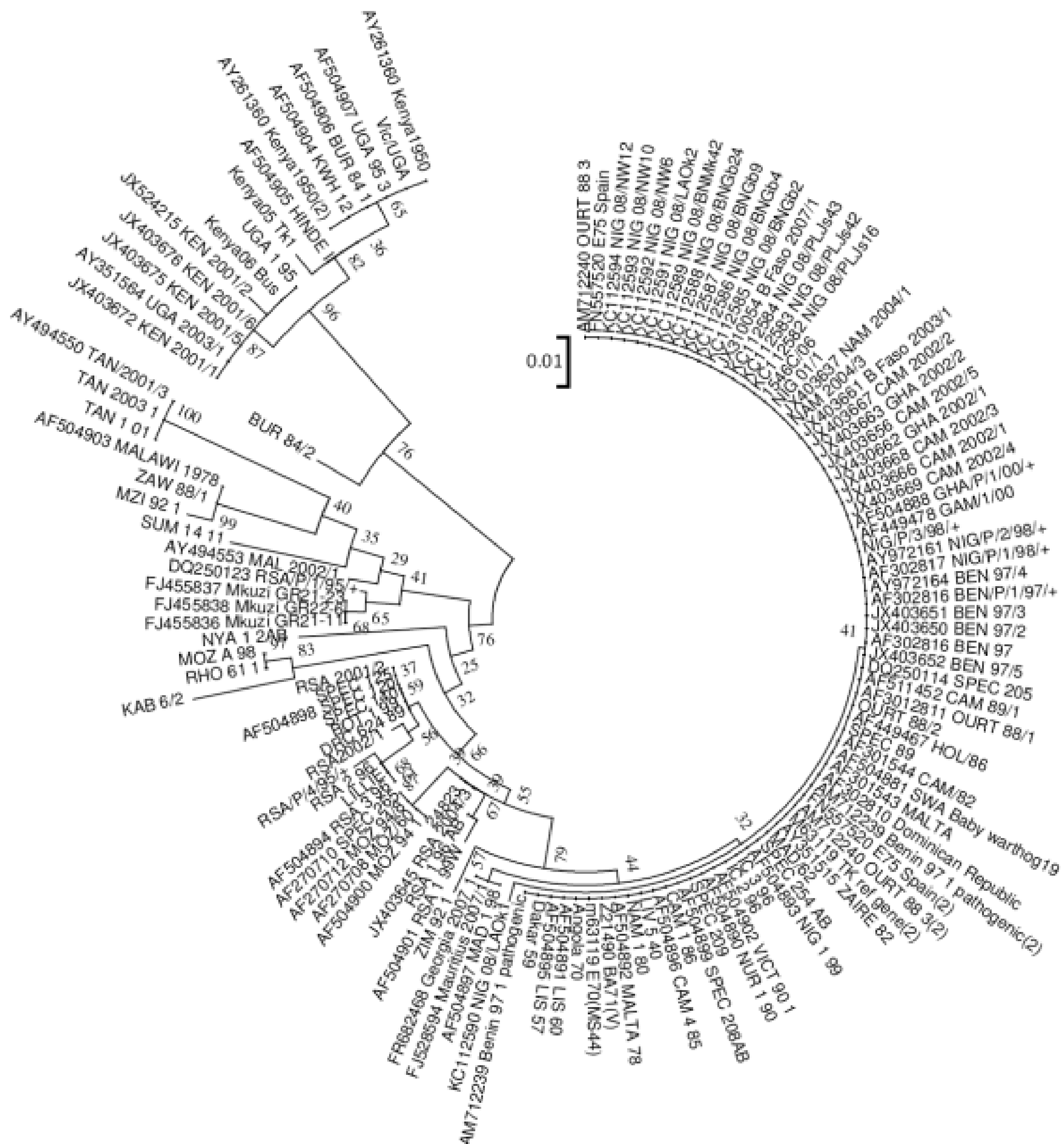


Fig. 7.2. Unrooted neighbour-joining tree depicting the Thymidine kinase amino acid sequence relationships of ASF viruses.

7.4 Discussion

Phylogenetic analysis of the TK gene revealed that the majority of viruses within the ESAC-WA p72 genotype (genotype I) have an identical *TK* gene product size of 196 amino acids while the ASF strains with truncated *TK* products, 185 amino acids in length, resulting from a frameshift mutation were primarily of East African origin. The large number of southern African ASF strains also possessed frameshift

mutations but a larger polypeptide of 188 amino acids was predicted for these. Previous work by Moore and colleagues [15] confirmed that the TK gene is involved in the virulence of ASFV (higher pathogenicity, shorter duration to death, shorter days to onset of clinical signs and viraemia from infection, longer duration of clinical signs, and higher titres of viraemia) [15].

Whether the truncated *TK* products will have a major effect on the virulence of the virus strains is not clear since no definitive pattern of pathogenicity exists between the isolates with truncated (185 or 188 amino acids) and those with full length *TK* (196 amino acids) products. However, all of the viruses with clearly identified truncated products, for which virulence data were available, were all classified as highly pathogenic isolates. Some viruses with 196aa were also highly pathogenic. Since *TK* is responsible for the catalysation of deoxythymidine monophosphate first reaction before it is further phosphorylated to deoxythymidine diphosphate by the enzyme thymidylate kinase which is later converted to deoxythymidine triphosphate by the enzyme nucleoside diphosphate kinase, it plays a very vital role in the DNA synthesis and rapid replications of the ASF virus *in vivo*. In view of the above, the truncation or extensive addition of nonsense gene to the TK gene length will affect the DNA production either by termination or reduction of speed of reactions.

Similarly, the East Africa isolates with the exception of the two Malawi strains (Malawi 1978 and Mal 2002_1), contained nonsense insertions of differing lengths at position 596. This contrasts with isolates from Europe, West and southern Africa which were all consistent in amplicon size and encoded a full-length protein. Since TK protein heterogeneity is unexpected in an enzyme with such an important function, it is predicted that these TK size differences may still have an effect on

virulence because it has been demonstrated that loss of the *TK* gene results in reduced virulence, low mortality and loss of pathogenicity [15].

An important geographical pattern which can be linked to the frameshift mutations and a shorter TK protein was revealed in this study. The East African viruses which are known to have high *p72* genotypic diversities, presumably arising from the ancient association between sylvatic cycle and hosts [1], also displayed high levels of TK length and sequence heterogeneity. It is probable that the epidemiology of ASF in East Africa in which the soft tick plays an important role, may be linked to the observed nonsense insertion and truncated *TK* products. However, no specific pattern exists with regards to the species of origin of the viruses [4, 5].

Strains from southern Africa are strongly supported as grouping with the strains of the ESAC-WA genotype (Figure 7.1 and 7.2), implying a shared evolutionary history. It is noted that none of these smaller *TK* gene products are found within strains of the ESAC-WA genotype, and all, with the exception of the Mkuze viruses (which are all linked to the *Ornithodoros* species) originated from East Africa. Since viral transmission within these two regions primarily involves a sylvatic cycle it is possible that there may be a link between the observed truncated *TK* products and the sylvatic cycle [4, 5]. In the alternative, a mutation to extend the open reading frame in the ESACWA isolates used in this study may have occur to give them a 196aa sequence.

Acknowledgements

We wish thank the Food and Agricultural Organization (FAO), International Livestock Research Institute (ILRI) for financial support, staff of CISA-INIA and TADP-OVI for provision of the isolates used in this study, and the late Mr. S.B. Phologane of the Transboundary Animal Disease Programme (formerly, Exotic

Diseases Division) of Onderstepoort Veterinary Institute for preparing some of the DNA extracts used in this study.

7.5 References

1. Bastos ADS, Penrith M-L, Crucière C, Edrich J, Hutchings G, Roger F, Couacy-Hymann E, Thomson GR: **Genotyping field strains of African swine fever virus by partial *p72* gene characterization**. *Archives Virol,* 2003, **148**: 693–706.
2. Boshoff CI, Bastos ADS, Gerber LJ, Vosloo W: **Genetic characterisation of African swine fever viruses from outbreaks in southern Africa (1973-1999)**. *Vet Microbiol* 2007, **121**: 45–55.
3. Dixon LK, Escribano JM, Martins C, Rock DL, Salas ML, Wilkinson PJ; In Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds): ***Virus Taxonomy**: VIIIth Report of the International Committee on Taxonomy of Viruses.* Elsevier/Academic Press, London, 135-143, 2005.
4. Kollnberger SD, Gutierrez-Castañeda B, Foster-Cuevas M, Corteyn A, Parkhouse RME: **Identification of the principal serological immunodeterminants of African swine fever virus by screening a virus cDNA library with antibody**. *J Gen Virol* 2002, **83**: 1331-1342.
5. Lubisi BA, Bastos AD, Dwarka RM, Vosloo W: **Molecular epidemiology of African swine fever in East Africa**. *Archives Virol* 2005, **150**: 2439–2452.
6. Penrith M-L, Thomson GR, Bastos ADS; In Coetzer JAW, Tustin RC (eds): *Infectious Diseases of Livestock*. Oxford University Press, Cape Town, 1087-1119, 2004.

7. Vial L, Wieland B, Jori F, Etter E, Dixon L, Roger F: **African swine fever virus DNA in soft ticks, Senegal**. *Emerg Infect Dis* 2007, **13**: 1928-1931.
8. Yáñez RJ, Rodriguez JM, Nogal ML, Yuste L, Enriquez C, Rodriguez JF, Viñuela: **Analysis of the complete nucleotide sequence of African swine fever virus**. *Virology* 1995, **208**: 249-278.
9. Polatnick J, Hess W: **Altered thymidine kinase activity in culture cells inoculated with African swine fever virus**. *Am J Vet Res* 1970, **31**: 1609–1613
10. Blasco R, Lopez-Otin C, Munoz M, Bockamp E, Simon-Mateo C, Vinuela E: **Sequence and evolutionary relationship of African swine fever virus thymidine kinase**. *Virology* 1990, **178**: 301-304.
11. Hernandez AM, Tabares E: **Expression and characterization of the thymidine kinase gene of African swine fever virus**. *J Virol* 1991, **65**: 1046-1052.
12. Yáñez RJ, Rodriguez JM, Rodriguez JF, Salas ML, Vinuela E: **African swine fever virus thymidylate kinase gene: sequence and transcriptional mapping**. *J Gen Virol* 1993, **74**: 1633-1638.
13. Cunha CV, Costa JV: **Induction of ribonucleic activity in cells infected with African swine fever virus**. *Virology* 1992, **187**: 73-83.
14. Tulman ER, Delhon GA, Ku BK, Rock DL: **African swine fever virus**. *Curr Top Microbiol Immunol.* 2009, **328**: 43-87.
15. Moore DM, Zsak L, Neilan JG, Lu Z, Rock DL: **The African swine fever virus thymidine kinase gene is required for efficient replication in swine macrophages and for virulence in swine**. *J Virol* 1998, **72**: 10310-10315.
16. Buller RML, Smith GL, Cremer K, Notkins AL, Moss B: **Decrease virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype**. *Nature* 1985, **317**: 813-815.

17. Kochneva GV, Urmanov IH, Ryabchikova EI, Streltsov VV, Serpinsky OI: **Fine mechanism of ectromelia virus thymidine kinase-negative mutant avirulence**. *Virus Res* 1994, 34: 49-61.
18. Field HJ, Wildy P: **The pathogenicity of thymidine kinase-deficient mutants of herpes simplex virus in mice**. *J Hyg (Lond)* 1978, **81**: 267-277.
19. Kit S, Qavi H, Dubbs DR, Otsuka H: **Attenuated marmoset herpesvirus isolated from recombinants of virulent marmoset herpesvirus and hybrid plasmids**. *J Med Virol* 1983, **12**: 25-36.
20. Plowright W, Thomson GR, Nesser JA; In Coetzer JAW, Thomson GR, Tustin RC (eds.): **African swine fever**: *Infectious Diseases of Livestock with Special Reference to Southern Africa.* Oxford University Press, Cape Town, London, New York, 567-599, 1994.
21. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-Van Dillen PME, Van Der Noordaa J: **Rapid and simple method for purification of nucleic acids**. *J Clin Microbiol* 1990, **28**: 495-503.
22. Edrich JL: *Genetic characterization of African swine fever viruses from Europe and West Africa*, M.Tech dissertation, Pretoria Technikon, 2002.
23. Rychlik W; In White BA (ed): **Selection of primers for polymerase chain reaction**. *PCR protocols: Current methods and applications.* Humana Press Inc, Totowa, NJ, 31-40, 1993.
24. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: **MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods**. *Mol Biol Evolution* 2011, **28**: 2731-2739.

25. Swofford DL: ***PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods).*** **Version 4.0b 10**. MA, Sinauer Associates, Inc., USA, 2002.

26. Hillis DM, Bull JJ: **An Empirical Test of Bootstrapping as a Method for Assessing Confidence in Phylogenetic Analysis**. *Syst Biol* 1993, **42**: 182-192.

27. Simmons MP, Ochoterena H: **Gaps as character in sequence-based phylogenetic analyses**. *Syst Biol* 2000, **49**: 369-381.

8.0 GENERAL DISCUSSION AND CONCLUSIONS

African swine fever remains a problem for the Nigerian pig industry. By extension, the extent of the infection in the country has been evaluated in this report and its wider effect within the West African subregion will need to be critically evaluated since Nigeria and other West and Central African countries operate a free-trade zone and animal health surveillance systems are weak. Though the surveillance done in this project was limited and carried out in selected locations and certain regions in Nigeria, and it appeared to have under-represented certain pig communities, the outcomes did however provide valuable new insights into the the Nigerian pig industry vis-a-vis that of transboundary infectious diseases like ASF in the country.

The sampling targeted areas of high pig concentrations because the distribution of pigs varies due to religious and cultural differences in human population. Within the target areas, the sampling was startified and randomised as described in previous chapters. Since the cooperation of ordinary farmers and farmer groups were used partially as the basis for sampling in this study and we are not unaware of any deficiencies that may attend such sampling, it was considered the best approach to ensure the widest form of sampling that can be presently be done pig farmers have great mistrust for government policies and have a general dissatisfaction with the government compensation/no-compensation policies. It is clear that the veterinary authorities will have to refine the current agricultural policies to enhance better government-farmer relationships as the control of infectious animal disease requires the cooperation of all stakeholders in order to be successful. A fair compensation scheme may also be considered for farmers that lose pigs or report lossess due to outbreaks of ASF.

To date, no stamping out has been carried out for outbreak(s) of ASF, however farmers were of the opinion that outbreaks should have been controlled by culling and compensation as was the case when outbreaks of highly pathogenic avian influenza H5N1 occurred in 2006-2008 (Joannis et al., 2008). It will also be valuable to establish a routine surveillance system for infectious and transboundary diseases of pigs. While this work has also proved that the spread of ASF continues to be an issue in Nigeria especially in locations with intensive pig activities, a review of the current animal surveillance system is advocated to enhance effectiveness. The strength of the project lies in the use of multiple testing systems to resolve the prevalence of ASF in Nigeria, but its weakness included those mentioned above as well as the risk of non-continuation of active surveillance programme in Nigeria.

It is known that certain farm practices and marketing systems may have enhanced the continued spread and infection of new/naïve pig farms in Nigeria based on discussions with farmers and an improved understanding of the pig farming system. One of the objectives of this study was therefore to analyse the risk factors and effectiveness of the biosecurity system in place. An evaluation of the risk of ASF infection on pig farms in Nigeria and an assessment of the effectiveness of self-reported biosecurity practices were performed. Certain risk factors were identified in Chapter 5 , including the presence of an abattoir in pig communities which may be associated with all or a combination of: (i) presentation of sick pigs for slaughter at the abattoirs, (ii) potential re-infection of farms by rats, wild birds and scavenging animals that scavenge around the open abattoir and (iii) inadvertent infection of farms by farmers; and the presence of an infected farm in a neighbourhood. Although, it is convenient to have pig slaughter facilities situated within the farm settlement as it reduces transport costs and stress to the animals, it is however desirable from the risk

estimation that the farm settlement be reorganised to exclude all pig slaughter slabs and abattoirs and that a separate location be created for such activities. Similarly, since neighbourhood effect was shown to be statistically significant as a risk for pig farm infection, and this may not be unconnected with the free-range pigs and current marketing structures/pig movement, efforts should be intensified to reduce the present networks, connectivity and neighbourhood-mediated effect of spread of ASF.

It is understood that peri-urban poverty remains a major issue in Nigeria and animal agriculture has been identified as one of the main means of combatting poverty in rural and urban areas. The study on biosecurity assessment and cost implications (Chapter) demonstrated that small-scale piggery operations are profitable albeit subject to various risks and uncertainties amongst which ASF is particularly important in West Africa. Since the education of pig farmers is important in the effort to reduce the burden of infection and possibly control ASF, and this work has demonstrated an economic approach to managing and preventing ASF virus infection at farm level, it will be apt to utilize this approach to convince farmers of the necessity and benefits of farm-based biosecurity.

Though the estimation of farm profitability may deviate from set standards based on optimum productivity, differential costing and valuations, risks and uncertainties, because interplay of various factors may affect farm operations and lower the obtained profitability from this report; overall a situation of ASF virus infection prevention at the level of farm-based biosecurity intervention will be more effective than taking no action since infection reproductive ratio can be significantly reduced and spread can also be minimized. ASF is currently endemic in Nigeria and in most of the West African states, and no vaccine is available to control the disease. Concentration of efforts on farm-level and community-based biosecurity is therefore

advocated to reduce the burden of infection. It has also been stated that this activity will impact positively on control of other transboundary animal diseases like foot-and-mouth disease (FMD), classical swine fever (CSF), Aujeszky's disease, swine vesicular disease (SVD), porcine circoviruses (PCV) and porcine reproductive and respiratory syndrome virus (PRRS).

It should be noted that the valuations and final costs arrived at in this study can have wider application for convincing farmers on adoption of biosecurity, and as a template for feasibility plans for setting up other pig projects since it is comprehensive and havs location-wide applications beyond the immediate use for benefit cost analysis.

Biosecurity should be combined with good farm management practices and farmers' cooperation for implementation should be encouraged. It must be noted that population of pigs has significantly dropped from the pre-1997 estimate of over 7 million to approximately 2 million heads of pigs since the advent of ASF in Nigeria and many persons are unwilling to get involved with this economic activity because of the risk of ASF infection. It should be possible to train extension agents, veterinarians and government agricultural workers to communicate the message of biosecurity, including its financial benefit to pig farmers. The use of community leaders and cooperative unions may also assist in this regard.

Aspects of biosecurity that will need to be intensified based on the analysis in this work include food and water control, the separation or isolation of sick pigs, and the washing and disinfection of farm tools and equipment as these were effective measures for reducing the of risk of ASF; however, since biosecurity principles do not work in isolation, these identified measures as well as others not listed will need to be implemented. It was also confirmed that for there to be an effective management and control of this disease, workers in the animal industry especially the farmers,

veterinarians and paraveterinarians need to carefully manage ASF outbreak situations using biocontainment and bioexclusion principles.

Since one of the objectives of this study was to evaluate the claims of farmers on the effectiveness of ethnoveterinary preparations, an *in vitro* evaluation was carried out (Chapter 6) which confirmed that farmers' claims of effectiveness of some plants in the treatment of ASF virus infection cannot be discarded in view of the results obtained. Though the present work is a preliminary evaluation of the plant *Ancistrocladus korupensis*, it should stimulate further research in the field of antivirals against ASF virus, particularly in view of the current absence of a vaccine or therapeutic agents. Future research on the *A. korupensis* plant evaluated in this study should focus on determining the particular metabolites or combinations of metabolites that are responsible for the therapeutic claims ascribed to this plant by the farmers, as well as on how to reduce toxicity.

In the final research chapter (Chapter 7), the interplay between host environment and pathogen prompted a preliminary molecular-based study into the possibility of identifying gene variants that are predictors or indicators of virulence. For this purpose, field strains predominantly of the ESAC-WA genotype (*p72* genotype I) and those representative of 20 other known genotypes were selected for characterisation. The results confirmed the existence of naturally occurring truncated TK products primarily within the East African region, but also within southern Africa. Although the size of the predicted TK protein varied between genotypes, the majority of genotype I isolates encoded the full-length 196 amino acid polypeptide. The smaller *TK* products of 185 and 188 amino acids, arising from a frameshift mutation at certain positions (e.g. in position 561 in UGA 95/3, KWH 12, BUR 84/1 and HINDE II) generally result in a stop codon immediately thereafter. Thus, it can be concluded that

although the TK gene does not appear to be a good predictor for strain pathogenicity it appears that truncated versions of the protein are associated with the presence of the sylvatic and domestic pig-tick cycle.

In conclusion, though this study has evaluated and arrived at important considerations for having a minimal disease-free and profitable pig production system in Nigeria, some analysis are beyond the immediate reach of this current project, for example an awareness of certain aspects of the epidemiology which has not been considered in this study including the pig movement and marketing structures and their associated network analysis. It will be important to carry out independent studies to evaluate these aspects more critically and use the outcome of this current work as a template to draft a comprehensive national health plan for the pig industry in Nigeria.

REFERENCES

[1]. Almazan, F., Rodriguez, J. M., Andres, G., Perez, R., Vinuela, E. and Rodriguez, J. F. (1992). Transcriptional analysis of multigene family 110 of African swine fever virus. *Journal of Virology,* 66: 6655-6667.

[2]. Agüero, M., J. Frenandez, L. Romero, C. Sanchez-Mascaraque, M. Arias, and J. M. Sanchez-Viscaino. (2003). Highly sensitive PCR assay for routine diagnosis of African swine fever virus in clinical samples. *Journal of Clinical Microbiology,* 41 (9), 4431-4434.

[3]. Akinyemi KO, Oladapo O, Okwara CE, Ibe CC, Fasure KA. (2005). Screening of crude extract of six medicinal plants used in South-West Nigerian unorthodox medicine for anti-methicillin resistant Staphylococcus aureus activity. *BMC Complementary and Alternative Medicine*, 5:6 doi:10.1186/1472-6882-5-6.

[4]. Anderson EC, Hutchings GH, Mukarati N, Wilkinson PJ. (1998). African swine fever virus infection of the bushpig (Potamochoerus porcus) and its significance in the epidemiology of the disease. *Veterinary Microbiology*, 62: 1-15.

[5]. Arnot LF, Du Toit JT, Bastos ADS. (2009). Molecular monitoring of African swine fever virus using surveys targeted at adult Ornithodoros ticks: a re-evaluation of Mkuze Game Reserve, South Africa. *Onderstepoort Journal of Veterinary Research*, 76: 385-392.

[6]. Babalobi, O. O., Olugasa, B. O. Oluwayelu, D. O. Ijagbone, I. F. Ayoade, G. O. and Agbede, S. A. (2007). Analysis and evaluation of mortality losses of the 2001 African swine fever outbreak, Ibadan, Nigeria. *Tropical Animal Health Production*, 39: 533–542.

[7]. Bala A, Daneji AI, Luther NJ, Chafe U, Fasina FO, Ifende VI, Emenna P, Bolajoko MP and Ubandawaki S. 2009. Survey of antibody to African swine fever virus (ASFV) in free rabge domestic pigs, Zuru Kebbi State. *Vom Journal of Veterinary Science* 6: 58-60.

[8]. Bastos, A.D.S., Penrith, M.-L., Crucière, C., Edrich, J., Hutchings, G., Roger, F., Couacy-Hymann, E. and Thomson, G.R. (2003). Genotyping field strains of African swine fever virus by partial *p72* gene characterization. *Archives of Virology* 148: 693–706.

[9]. Bastos, A. D. S., Penrith, M. L., Macome, F., Pinto, F. and Thomson, G. R. (2004). Co-circulation of two genetically distinct viruses in an outbreak of African swine fever in Mozambique: no evidence for individual co-infection. *Veterinary Microbiology*, 103, 169-182.

[10]. Bech-Nielsen S, Bonilla QP, Sanchez-Viscaino JM. (1993). Benefit-cost analysis of the current African swine fever eradication program in Spain and of an accelerated program. *Preventive Veterinary Medicine*, 17:235-249.

[11]. Bentley KW. (1996). *β*-Phenylethylamines and the isoquinoline alkaloids. *Natural Product Reports*, 13: 127-150.

[12]. Biront, P., Castryck, F. and Leunen, J. (1987). An epizootic of African swine fever in Belgium and its eradication. *Veterinary Record*, 120: 432-434.

[13]. Blasco, R., de la Vega, I., Almazan, F., Aguero, M., Vinuela, E. (1989). Genetic variation of African swine fever virus: variable regions near the ends of the viral DNA. *Virology,* 173: 251–257.

[14]. Blasco R, Lopez-Otin C, Munoz M, Bockamp E, Simon-Mateo C, Vinuela E. (1990). Sequence and evolutionary relationship of African swine fever virus thymidine kinase. *Virology*, 178: 301-304.

[15]. Boklund, A., Alban, L., Mortensen, S., Houe, H. (2004). Biosecurity in 116 Danish fattening swineherds: descriptive results and factor analysis. *Preventive Veterinary Medicine*, 66: 49–62.

[16]. Boklund, A., Mortensen, S., Houe, H. (2005). Biosecurity in 121 Danish sow herds. *Acta Veterinaria Scandinavica*. Supplement. 100: 5-14.

[17]. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-Van Dillen PME, Van Der Noordaa J. (1990). Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology*, 28: 495-503.

[18]. Boshoff, C.I., Bastos, A.D.S. Gerber, L.J. and Vosloo, W. (2007). Genetic characterisation of African swine fever viruses from outbreaks in southern Africa (1973-1999). *Veterinary Microbiology*, 121: 45–55.

[19]. Boyd, M. R., Hallock, Y. F., Cardelinna, J. H., Manfredi, K. P., Blunt, J. W., McMahon, J. B., Buckheit, Jr, R. W., Bringham, G., Schaffer, M., Cragg, G. M., Thomas, D. W. and Jato, J. G. (1994). Anti-HIV Michellamines from *Ancistrocladus korupensis*. *Journal of Medicinal Chemistry*, 37:1740-1745.

[20]. Bram, R. A., George, J. E., Reichard, R. E.and Tachnick, W. J. (2002). Threat of foreign arthropod-borne pathogens to livestock in the United States. *Journal of Medical Entomology*, 39(3): 405-416.

[21]. Buller RML, Smith GL, Cremer K, Notkins AL, Moss B. (1985). Decrease virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature*, 317: 813-815.

[22]. Callaway, E. (2012). Pig fever sweeps across Russia: Deadly virus may be poised to spread to neighbouring states. Nature, 488: 565-566. Doi:10.1038/488565a.

[23]. Carr, J. (1998). Farms targets per 100 sows. In: *Garth Pig Stockmanship Standards.* 5M Enterprises Ltd., Sheffield, UK.

[24]. Carrascosa, A. L., Bustos, M. J., de Leon, P. (2011). Methods for growing and titrating African swine fever virus: field and laboratory samples. *Curr Protoc Cell Biol.* Chapter 26: Unit 26.14. doi: 10.1002/0471143030.cb2614s53.

[25]. Casal J, de Manuel A, Mateu E, Martin M. (2007). Biosecurity measures on swine farms in Spain: Perceptions by farmers and their relationship to current on-farm measures. *Preventive Veterinary Medicine*, 82:138-150

[26]. Chaber A-L, Allebone-Webb S, Lignereux Y, Cunningham AA, Rowcliffe JM. (2010). The scale of illegal meat importation from Africa to Europe via Paris. *Conservation Letters*, doi: 10.1111/j.1755-263X.2010.00121.x

[27]. Chapman, D. A., Tcherepanov, V., Upton, C., Dixon, L.K. (2008). Comparison of the genome sequences of non-pathogenic and pathogenic African swine fever virus isolates. *Journal of General Virology,* 89: 397–408.

[28]. Chapman, D. A. G., Darby, A. C., Da Silva, M., Upton, C., Radford, A. D., Dixon, L. K. (2011). Genomic analysis of highly virulent isolate of african swine fever virus. *Emerging Infectious Diseases*. DOI: 10.3201/eid1704.101283

[29]. Choi YH, Kim HK, Verpoort R. (2007). Metabolomics, In: Kayser O, Quax WJ, (eds.), *Medicinal Plant Biotechnology: From Basic Research to Industrial Application* (Volume

1), Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim, Germany, pp 9-28. Available online at: http://onlinelibrary.wiley.com/book/10.1002/9783527619771, 2007.

[30]. CIA World Factbook- Benin. (2006). Available at: http://www.cia.gov/library/publications/the-world-factbook/geos/bn.html . Accessed on 7th March, 2010.

[31]. Costard S, Porphyre V, Messad S, Rakotondrahanta S, Vidon H, Roger F, Pfeiffer DU. (2009). Multivariate analysis of management and biosecurity practices in smallholder pig farms in Madagascar. *Preventive Veterinary Medicine*, 92: 199-209.

[32]. Costard, S., Weiland, B., de Glanville, W., Jori, F., Rowlands, R., Vosloo, W., Roger, F., Pfeiffer, D.U., Dixon, L.K. (2009b). African swine fever: how can global spread be prevented? *Philosophical Transactions of the Royal Society B*, 364, 2683-2696.

[33]. Cragg GM, Newman DJ: Drug discovery and development from natural products: The way forward. (2005). *Proceedings of the 11th Natural Product Research for Eastern and Central Africa (NAPRECA) Symposium,* 9-12 August 2005, Hôtel Panorama, Antananarivo, Madagascar, 11: 56-69.

[34]. Cunha CV, Costa JV. (1992). Induction of ribonucleic activity in cells infected with African swine fever virus. *Virology*, 187: 73-83.

[35]. de Kock, G., Robinson, E. M. and Keppel, J. J. G. (1940). Swine fever in South Africa. *Onderstepoort Journal of Veterinary Science and Animal Industry,* 14: 31-93.

[36]. Dexter DL, Hesson DP, Ardecky RJ, Rao GV, Tippett DL, Dusak BA, Paull KD, Plowman J, DeLarco BM, Narayanan VL, Forbes M. (1985). Activity of a novel 4-quinolinecarbocylic acid, NSC 368390 [6-fluoro-2-(2′ fluoro-1,1′-biphenyl-4-yl)-3-methyl-4-quinolinecarboxylic acid sodium salt] against experimental tumours. *Cancer Research*, 45: 5563-5568.

[37]. de Villiers, E. P., Gallardo, C., Arias, M., da Silva, M., Upton, C., Martin, R and Bishop, R. P. (2010) Phylogenomic analysis of 11 complete African swine fever virus genome sequences., *Virology*, 400(1):128-136.

[38]. Dixon, L. K., Bristow, C., Wilkinson, P. J. and Sumption, K. J. (1990). Identification of a variable region of the African swine fever virus genome that has undergone separate

DNA rearrangements leading to expansion of minisatellite-like sequences. *Journal of Molecular Biology*, 216:677-688.

[39]. Dixon, L. K., S. R. F. Twigg, S. A. Baylis, S. Vydelingum, C. Bristow, J. M. Hammond, G. L. Smith. (1994). Nucleotide sequence of a 55 kbp region from the right end of the genome of a pathogenic African swine fever virus isolate (Malawi LIL20/1). *Journal of General Virology,* 75:1655-1684.

[40]. Dixon, L. K., Escribano, J. M., Martins, C., Rock, D. L., Salas, M. L. and Wilkinson, P. J. (2005). *Asfarviridae.* In: Fauquet, C. M., M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball (eds), *Virus Taxonomy, VIIIth Report of the International Committee on Taxonomy of Viruses*, pp. 135–143. Elsevier Academic Press, London.

[41]. Edelsten RM, Chinombo DO (1995). An outbreak of African swine fever in the Southern region of Malawi. *Revue Scientifique et Technique, Office International des Épizooties* 14(3): 655–666

[42]. Ekue FN, Tanya VN (1985). The 1982 African swine fever epizootic in Cameroun. *Revue Scientifique et Technique, Office International des Épizooties* 1: 65–70

[43]. Ekue N.F. and Wilkinson P.J. (1990). Absence of *Ornithodoros moubata*, the vector of African swine fever virus, from the main pig producing area of Cameroon. *Tropical Animal Health and Production*, 22 (2): 127-131.

[44]. El-Hicheri K., (1998). Emergency Assistance on control and eradication of an outbreak of African swine fever in Western Nigeria. *Report of the FAO Consultancy Mission to Nigeria.* TCP/NIR/7822(E). FAO, Rome, December, 1998.

[45]. El-Hicheri K., Gomez-Tejedor C., Penrith M.-L., Davies G., Douati A., Edoukou G.J. and Wojciechowski W. (1998). – L'épizootie de peste porcine africaine de 1996 en Côte d'Ivoire. *Rev. sci. tech. Off. int. Epiz.,* 17 (3): 660-673.

[46]. Ellis PR, Putt SN. (1981). The Epidemiological and Economic Implications of Foot-and-Mouth disease Vaccination Programme in Kenya. Consultancy Report to the Government of Kenya.

[47]. European Food Safety Authority (EFSA), 2009: Scientific review on African swine fever. Available at http://www.efsa.europa.eu/en/supporting/pub/5e.htm (accessed February 22, 2012).

[48]. EFSA Panel on Animal Health and Welfare. (2010). Scientific Opinion on African swine fever. *EFSA Journal*, 8(3): 149 pp. doi:10.2903/j.efsa.2010.1556. Available online at: www.efsa.europa.eu.

[49]. Etter EMC, Seck I, Grosbois V, Jori F, Blanco E, Vial L, Akakpo AJ, Bada-Alhambedji R, Kone P, Roger FL. (2011). Seroprevalence of African swine fever in Senegal, 2006. *Emerging Infectious Diseases*, 17(1): 49-54.

[50]. Fadiga, M., Jost C and Ihedioha J. (2011). Financial costs of disease burden, morbidity and mortality from priority livestock diseases in Nigeria: Disease burden and cost-benefit analysis of targeted intervention. Available at: http://www.ilri.org/content/financial-costs-disease-burden-morbidity-and-mortality-priority-livestock-diseases-nigeria. Accessed on 5 March, 2012.

[51]. Fasina, F.O., D. ShamakiA.A. Makinde, and L.H. Lombin; ASF PITT, 2009: Field surveillance and laboratory diagnoses of African swine fever in Nigeria. *International Symposium on Sustainable Improvement of Animal Production and Health*. Vienna Austria, IAEA-CN-174-151. Pp300-302. Also available at: http://www-naweb.iaea.org/nafa/aph/BookOfExtendedSynopses.pdf .

[52]. Fasina, F. O., D. Shamaki, A. A. Makinde, L. H. Lombin, D. D. Lazarus, S. A. Rufai, S. S. Adamu, D. Agom, V. Pelayo, A. Soler, A. Simón, A. J. Adedeji, M. B. Yakubu, S. Mantip, A. J. Benshak, I. Okeke, P. Anagor, D. C. Mandeng, B. O. Akanbi, A. A. Ajibade, I. Faramade, M. M. Kazeem, L. U. Enurah, R. Bishop, R. Anchuelo, J. H. Martin, C. Gallardo. (2010). Surveillance for African Swine Fever in Nigeria, 2006-2009. *Transboundary and Emerging Diseases,* 57(4): 244-253.

[53]. Field HJ, Wildy P. (1978). The pathogenicity of thymidine kinase-deficient mutants of herpes simplex virus in mice. *Journal of Hygiene (Lond)*, 81: 267-277.

[54]. Firestone, S.M., Schemann, K.A., Toribio, J-A.L.M.L., Ward, M.P., Dhand, N.K. (2011). A case-control study of risk factors for equine influenza spread onto horse premises during the 2007 epidemic in Australia. *Preventive Veterinary Medicine*, 100, 53–63.

[55]. Food and Agriculture Organization of the United Nations (FAO). (2001). Economic impacts of transboundary pests and diseases (Part III). In: *The State of Food and Agriculture*, 2001. Available online at http://www.fao.org/docrep/003/x9800e/x9800e16.htm#TopOfPage.

[56]. Food and Agriculture Organization of the United Nations (FAO) (2009). African swine fever spread in the Russian Federation and the risk for the region. EMPRES WATCH, December 2009. Available online at: ftp://ftp.fao.org/docrep/fao/012/ak718e/ak718e00.pdf . Accessed on 6th March, 2010.

[57]. Food and Agriculture Organization of the United Nations/World Organisation for Animal Health/World Bank. (2008). Biosecurity for highly pathogenic avian influenza: Issues and options. FAO, Rome, 73pp

[58]. Food and Agriculture Organization of the United Nations/World Organisation for Animal Health/World Bank. (2010). *Good practices for biosecurity in the pig sector: Issues and options in developing and transition countries*. FAO Animal Production and Health Paper No. 169. Rome, FAO.

[59]. Foster, P. F. and Sork, V. L. (1997). Population and genetic structure of the West African rain forest Liana *Ancistrocladus korupensis* (Ancistrocladaceae). *American Journal of Botany,* 84:1078-1091.

[60]. Fournie, G., Rushton, J., Pfeiffer, D. (2012). Disease modelling and the human factor. Veterinary Record, 170: 157-158. doi: 10:1136/vr.e588.

[61]. Gago da Câmara N J 1932 História da peste suína em Angola. *Pecuária* 1: 25–40.

[62]. Gallardo, C. and M. Arias. (2009). *ASF Diagnosis Protocols*. CISA-INIA, Madrid, Spain

[63]. Gallardo, C., Mwaengo, D.M., MacHaria, J.M., Arias, M., Taracha, E.A., Soler, A., Okoth, E., Martin, E., Kasiti, J. and Bishop. R.P. (2009). Enhanced discrimination of African swine fever virus isolates through nucleotide sequencing of the p54, p72, and pB602L (CVR) genes. *Virus Genes* 38: 85–95.

[64]. Gallardo, C., Ademun, A.R., Nieto, R., Nantima, N., Arias, M., Martin, E., Pelayo, V., Bishop, R.P. (2011). Genotyping of African swine fever virus (ASFV) isolates associated with disease outbreaks in Uganda in 2007. *African Journal of Biotechnology*, 10(17), 3488–3497.

[65]. Gallardo C, Okoth E, Pelayo V, Anchuelo R, Martín E, Simón A, Llorente A, Nieto R, Soler A, Martín R, Arias M, Bishop RP. (2011). African swine fever viruses (ASFV) with two different genotypes, both of which occur in domestic pigs, are associated with ticks and adult warthogs, respectively, at a single geographical site. *Journal of General Virology*, 92: 432-444.

[66]. Galindo I, Hernaez B, Berna J, Fenoll J, Cenis JL, Escribano JM, Alonso C. (2011). Comparative inhibitory activity of the stilbenes resveratrol and oxyresveratrol on African swine fever virus replication. *Antiviral Research,* 91: 57-63.

[67]. Gardner IA, Willeberg P, Mousing J. (2002). Empirical and theoretical evidence for herd size as a risk factor for swine diseases. *Animal Health Research Reviews*, 3: 43-55.

[68]. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., Bairoch, A. (2005). Protein identification and analysis tools on the ExPASY Server. In: Walker, J. M. (ed.), *The Proteomics Protocols Handbook*. Humana Press, pp 571-607.

[69]. Goebel SJ, Johnson GP, Perkus ME, Davis SW, Winslow JP, Paoletti E. (1990). The complete DNA sequence of vaccinia virus. *Virology*, 179: 247-266.

[70]. Gomez-Puertas P, Rodriguez F, Oviedo JM, Ramiro-Ibanez F, Ruiz-Gonzalvo F, Alonso C, Escribano JM. 1996 Neutralizing antibodies to different proteins of African swine fever virus inhibit both virus attachment and internalization. *Journal of Virology* 70: 5689–5694.

[71]. Gonzague M, Roger F, Bastos A, Burger C, Randriamparany T, Smondack S, Cruciere C. (2001). Isolation of a non-haemadsorbing, non cytopathic strain of African swine fever virus in Madagascar. *Epidemiology and Infection*, 126:453-459.

[72]. Guo L, Qian JY, Abrams R, Tang HM, Weiser T, Sanders MJ, Kolaja KL. (2011). The electrophysiological effects of cardiac glycosides in human iPSC-derived cardiomyocytes and in guinea pig isolated hearts. *Cellular Physiology Biochemistry*, 27: 453-462.

[73]. Hallock YF, Manfredi KP, Blunt JW, Cardelinna JH, Schaffer M, Gulden K-P, Bringmann G, Lee AY, Clardy J, Francois G, Boyd MR. (1994). Korupensamine A-D, novel antimalarial alkaloids from Ancistrocladus korupensis. *Journal of Organic Chemistry,* 59: 6349-6355.

[74]. Harborne JB. (1973). *Phytochemical Methods*. Chapman and Hall, New York, NY, USA.

[75]. Haresnape JM, Wilkinson PJ. (1989). A study of African swine fever virus infected ticks(Ornithodoros moubata) collected from three villages in the ASF enzootic area of

Malawi following an outbreak of the disease in domestic pigs. Epidemiology and Infection, 102: 507-522.

[76]. Haresnape JM, Wilkinson PJ, Mellor PS (1988). Isolation of African swine fever virus from ticks of the Ornithodoros moubata complex (Ixodoidea: Argasidae) collected within the African swine fever enzootic area of Malawi. Epidemiology and Infection, 101: 173-185

[77]. Harley EH. (2001). DAPSA: *DNA and protein sequence analysis,* Version 4.9. Department of Chemical Pathology, University of Cape Town, South Africa, 2001.

[78]. Hernandez AM, Tabares E. (1991). Expression and characterization of the thymidine kinase gene of African swine fever virus. *Journal of Virology*, 65: 1046-1052.

[79]. Hillis DM, Bull JJ. (1993). An Empirical Test of Bootstrapping as a Method for Assessing Confidence in Phylogenetic Analysis. *Systematic Biology*, 42: 182-192.

[80]. Horst, H.S., Huirne, R.R., Dijkhuizen, A.A. (1997). Risks and economic consequences of introducing classical swine fever into The Netherlands by feeding swill to swine. *Revue Scientifique et Technique, Office International des Épizooties,* 16, 207–214.

[81]. Hosmer, D.W., Lemeshow, S. (1989). Logistic regression for matched case-control studies. In: Hosmer, D.W., Lemeshow, S. (Eds.), *Applied Logistic Regression*. Wiley, New York, pp. 187–215.

[82]. Heuschele W.P. and Coggins, L. (1965). Isolation of African swine fever from a giant forest hog. *Bulletin of Epizootic Diseases in Africa*, 13: 255-256.

[83]. Irusta, P.M., Borca, M.V., Kutish, G.F., Lu, Z., Caler, E., Carrillo, C., Rock, D.L., (1996). Amino acid tandem repeats within a late viral gene define the central variable region of African swine fever virus. *Virology* 220: 20–27.

[84]. International Committee on Taxonomy of Viruses (ICTV) (2002). African swine fever virion section with lipid membranes, capsid and nucleoprotein core. Academic Press (2000). Available at: http://ictvdb.bio-mirror.cn/WIntkey/Images/em_asfar_ICTV.htm. Accessed on 4th March, 2012.

[85]. ICTVdB Management (2006). 00.002.0.01. Asfivirus. In: *ICTVdB - The Universal Virus Database*, version 4. Büchen-Osmond, C. (Ed), Columbia University, New York, USA. Also available at: http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/.

[86]. Ivanov V, Sandell E. (1992). Characterization of Polychlorinated Biphenyl Isomers in Sovol and Trichlorodiphenyl formulations by high-resolution Gas Chromatography with Electron Capture detection and high-resolution Gas Chromatography-Mass Spectrometry techniques. *Environmental Science and Technology*, 26: 2012-2017.

[87]. Joannis TM., Meseko AC., Oladokun AT., Ularamu HG., Egbuji NA., Solomon P, Nyam DC., Gado AD., Luka P, Ogedengbe EM., Yakubu MB., Tyem AD., Akinyede O, Shittu AI., Sulaiman KL., Owolodun O, Olawuyi, AK., Obishakin ET., Fasina FO. (2008). Serologic and virologic surveillance of avian influenza in Nigeria, 2006-7. *Eurosurveillance,* 2008, Vol. 13, Issue 42, pii=19007; Article 5, 13(40-51) : 608-612

[88]. Jori, F. and Bastos, A. D. S. (2009). Role of wild suids in the epidemiology of African swine fever. *EcoHealth,* 6(2):293-310.

[89]. Kelly GS. (1999). Squalene and its potential clinical uses. *Alternative Medicine Reviews*, 4: 29-36.

[90]. King DP, Reid SM, Hutchings GF, Grierson SS, Wilkinson PJ, Dixon LK, Bastos ADS, Drew TW. (2003). Development of a TaqMan PCR assay with internal amplification control for the detection of African swine fever virus. *Journal of Virological Methods*, 107: 53-61.

[91]. King K, Chapman D, Argilaguet JM, Fishbourne E, Hutet E, Cariolet R, Hutchings G, Oura CAL, Netherton CL, Moffat K, Taylor G, Le Potier M-F, Dixon LK, Takamatsu H-H. (2011). Protection of European domestic pigs from virulent African isolates of African swine fever by experimental immunisation. *Vaccine* 29: 4593-4600.

[92]. Kit S, Qavi H, Dubbs DR, Otsuka H. (1983). Attenuated marmoset herpesvirus isolated from recombinants of virulent marmoset herpesvirus and hybrid plasmids. *Journal of Medical Virology*, 12: 25-36.

[93]. Kollnberger SD, Gutierrez-Castañeda B, Foster-Cuevas M, Corteyn A, Parkhouse RME. (2002). Identification of the principal serological immunodeterminants of African swine

fever virus by screening a virus cDNA library with antibody. *Journal of General Virology*, 83: 1331-1342.

[94]. Kochneva GV, Urmanov IH, Ryabchikova EI, Streltsov VV, Serpinsky OI. (1994). Fine mechanism of ectromelia virus thymidine kinase-negative mutant avirulence. *Virus Research* 1994, 34: 49-61.

[95]. Kotze M, Eloff JN. (2002). Extraction of antibacterial compounds from *Combretum microphyllum* (Combetaceae). *South African Journal of Botany*, 68: 62-67.

[96]. Kudi AC, Myint SH. (1999). Antiviral activity of some Nigerian medicinal plant extracts. *Journal of Ethnopharmacology*, 68: 289-294.

[97]. Kudi AC, Umoh JU, Eduvie LO, Gefu J. (1999). Screening of some Nigerian medicinal plants for antibacterial activity. *Journal of Ethnopharmacology*, 67: 225-228.

[98]. Laird S, Lisinge E. (2012). Case studies from Cameroon: Contrasting benefit-sharing in the pharmaceutical and phytomedical industries. Case studies submitted to the CBD Secretariat, UNEP/CBD/COP/4/Inf.25, – 49 pp, Available online at http://www.biodiv.org/doc/case-studies/cs-abs-aristo.pdf, 1998, Accessed on 21st June, 2012.

[99]. Laird SA (2012). The Convention on Biological Diversity: changing ethical and legal frameworks for biodiversity research and prospecting. *Unasylva* 2003- *206 - Global Conventions Related to Forests*, 52: 2001/3, Available online at: http://www.fao.org/docrep/003/y1237e/y1237e00.htm#TopOfPage, , Accessed on 21st June, 2012.

[100]. Lambert M-E, D'Allaire S. (2009). Biosecurity in swine production: Widespread concerns? Presentation at the Banff Pork Seminar, Montreal. Available online at: http://www.thepigsite.com/articles/2928/biosecurity-in-swine-production-widespread-concerns .

[101]. Leeson, H.S. (1958). The recorded distribution of *Ornithodoros moubata* (Murray) (Acarina). *Bulletin of Entomological Research* 43: 407 – 411.

[102]. LeFevre PC (1998). African swine fever in West Africa: Togo, Senegal, Gambia and Guinea Bissau. Consultancy report submitted to FAO. Available at: http://www.fao.org/docrep/field/382399.htm. Accessed on 6th March, 2012.

[103]. Lewis, T., Zsak, L., Burrage, T.G., Lu, Z., Kutish, G.F., Neilan, J.G., Rock, D.L. (2000). An African swine fever virus *ERV1-ALR* Homologue, *9GL*, affects virion maturation and viral growth in macrophages and viral virulence in swine. J. Virol., 74(3):1275-1285.

[104]. Lubisi, B.A., Bastos, A.D. Dwarka, R.M. and Vosloo, W. (2005). Molecular epidemiology of African swine fever in East Africa. *Archives of Virology* 150: 2439–2452.

[105]. Lubisi, B.A., Bastos, A.D. Dwarka, R.M. and Vosloo, W. (2007). Intra-genotypic resolution of African swine fever viruses from an East African domestic pig cycle: a combined p72-CVR approach. *Virus Genes* 35: 729–735.

[106]. Lubisi B.A., Dwarka R.M., Meenowa D. and Jaumally R. (2009). An Investigation into the First Outbreak of African swine fever in the Republic of Mauritius. *Transboundary and Emerging Diseases* 56: 178–188.

[107]. Luther NJ, Umoh JI, Majiyagbe KA, Shamaki D and Udeani TKC. (2002). Incidence of antibody to African swine fever virus (ASFV) in pigs from Plateau State correlated with signs of African swine fever-like illness. *Proceedings of the 39th NVMA Annual Conference*, Sokoto, Nigeria. 144–146.

[108]. Luther, N.J., K.A. Majiyagbe, C.I. Nwosuh, D. Shamaki. (2007a). The epizootiology, prevalence and economic aspects of African swine fever in parts of Middle Belt central states of Nigeria. *Tropical Veterinarian* 25, 96-100.

[109]. Luther, N.J., D. Shamaki, K.A.Majiyagbe, C.I. Nwosuh, 2007b: The prevalence of African swine fever in parts of the middle belt central states of Nigeria. *Bulletin of Animal Production and Health in Africa,* 55, 79-81.

[110]. Luther, N.J., Majiyagbe, K.A., Shamaki, D., Lombin, L.H., Antiabong, J.F., Bitrus, Y. and Owolodun O., (2007a). Detection of African swine fever virus genomic DNA in a Nigerian red river hog (*Potamochoerus porcus*). *Veterinary Record,* 160 (2): 58-9.

[111]. Luther, N. J., Udeama, P. G., Majiyagbe, K. A., Shamaki, D., Antiabong, J., Bitrus, Y., Nwosuh, C. I. and Owolodun, O. A. (2007b). Polymerase Chain Reaction (PCR) detection of the genome of African Swine Fever Virus (ASFV). From natural infection in

a Nigerian baby warthog (*Phacochoereus aethiopicus*). *Nigerian Veterinary Journal* 28: 63–67.

[112]. Madec F, Rose N. (2003). How husbandry practices may contribute to the course of infectious diseases in pigs. *4th International Symposium on Emerging and Re-emerging Pig Diseases,* Rome June 29th – July 2nd, 2003.

[113]. Madureira AM, Ascenso JR, Valdeira L, Duarte A, Frade JP, Freitas G, Ferreira MJU. (2003). Evaluation of the antiviral and antimicrobial activities of Triterpenes isolated from *Euphorbia segetalis*. *Natural Product Research*, 17: 375-380.

[114]. Majiyagbe KA, Shamaki D, Luther NJ and Udeani TKC. 2004. African swine fever epidemics. The Nigerian experience, 1997–2004. *Vom Journal of Veterinary Science* 1: 138 –147.

[115]. Malmquist W.A. and Hay D. (1960). Hemadsorption and cytopathic effect produced by African swine fever virus in swine bone marrow and buffy coat cultures. *American Journal of Veterinary Research*, 21: 104-108.

[116]. Mannelli, A., Sotgia, S., Patta, C., Sarria, A., Madrau, P., Sanna, L., Firinu, A., Laddomada, A. (1997). Effect of husbandry methods on seropositivity to African swine fever virus in Sardinian swine herds. *Preventive Veteinary Medicine,* 32: 235–241.

[117]. Mannelli, A., Sotgia, S., Patta, C., Oggiano, A., Carboni, A., Cossu, P., Laddomada, A. (1998). Temporal and spatial patterns of African swine fever in Sardinia. *Preventive Veteinary Medicine*, 35: 297-306.

[118]. Manso Ribeiro, J.J., and Azevedo, J.A. (1961). La peste porcine Africaine au Portugal. *Bull. Off. Int. Epiz.* 55, 88–108.

[119]. Marsh, W. (1999). The economics of animal health in farmed livestock at the herd level. *Rev. Sci. Tech. Off. Int. Epizoot.*, 18: 357–366.

[120]. McDaniel, H.A. (1986). African swine fever. In: Leman Glock, A. D., Mengeling, R. D.W.L., Penny, R.H.C., Scholl, E. and Straw, B. (eds), Diseases of Swine, 6th Ed., Ames, Iowa, USA. Iowa State University Press. p 237-245.

[121]. McDermott JJ, Randolph TF, Staal SJ. (1999). The economics of optimal health aand productivity in smallholder livestock systems in developing countries. *Rev. Sci. Tech. Off. Int. Epizoot.*, 18: 399–424.

[122]. McKercher PD, Hess WR and Hamdy F (1978). Residual viruses in pork products. *Applied Environmental Microbiology* **35**: 142–145.

[123]. McMahon, J. B., Currens, M. J., Gulakowski, R. J., Buckheit, Jr., R. W., Lackman-Smith, C., Hallock, Y. F., and Boyd, M. R. (1995). Michellamine B, a Novel Plant Alkaloid, Inhibits Human Immunodeficiency Virus-Induced Cell Killing by at Least Two Distinct Mechanisms. *Antimicrobial Agents and Chemotherapy*, 39: 484–488.

[124]. Mebus C.A., House C., Ruiz Gonzalvo F., Pineda J.M., Tapiador J., Pire J.J., Bergada J. and Yedloutschnig R.J. (1993). Survival of foot and mouth disease, African swine fever, and hog cholera viruses in Spanish Serrano cured hams and Iberian cured hams; shoulders and loins. *Food Microbiol.*, 10, 133-143.

[125]. Miller H.S. (1996). African swine fever poses risk to US hogs. *Agric. Res*., 44: 14-17.

[126]. Montgomery, R.E. (1921). On a form of swine fever occurring in British East Africa (Kenya Colony). *Journal of Comparative Pathology* 34: 159–191.

[127]. Moore, D.M., Zsak, L., Neilan, J.G., Lu, Z., Rock D. L. (1998). The African swine fever virus Thymidine Kinase gene is required for efficient replication in swine macrophages and for virulence in swine. J. Virol., 72 (12):10310-10315.

[128]. Moore, J.R. (2007). Swine production: a global perspective. Available at: http://en.engormix.com/MA-pig-industry/management/articles/swine-production-global-perspective_336.htm. Accessed on 6th March, 2010.

[129]. Mur, L., Martinez-Lopez, B., Sanchez-Viscaino, J. M. (2012). Risk of African swine fever introduction into the European Union through transport-associated routes: returning trucks and waste from international ships and planes. *BMC Veterinary Research*, 8:149. doi:10.1186/1746-6148-8-149

[130]. National Institute of Standards-USA (NIST). (2010). *Standard Reference Data*. Available online at http://www.nist.gov/srd/, Accessed on 15th June, 2010.

[131]. National Pork Producers Council. (2010). *Capital Pork Report-2010*. Available at http://npc.org/uploadedfiles/cprJAN-5.pdf . Accessed on 7th March, 2010.

[132]. Neilan J G, Zsak L, Lu Z, Burrage T G, Kutish G F, Rock D L 2004 Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection. *Virology* 319: 337–342.

[133]. Nespeca, R., Vaillancourt, J., Morrow, W.E.M., 1997. Validation of a poultry biosecurity survey. *Preventive Veterinary Medicine*, 31: 73–86.

[134]. Niessen WMA. (2001). *Current Practice of Gas Chromatography-Mass Spectrometry*. Marcel Dekker, CRC Press, New York, 2001.

[135]. Nix, R.J., Gallardo, C., Hutchings, G., Blanco, E., Dixon, L.K., (2006). Molecular epidemiology of African swine fever virus studied by analysis of four variable genome regions. *Archives of Virology* 151: 2475–2494.

[136]. Noremark M, Frossling J, Lewerin SS. (2010). Application of routines that contribute to on-farm biosecurity as reported by Swedish livestock farmers. *Transboundary and Emerging Diseases*, 57:225-236.

[137]. Nwanta JA, Shoyinka SVO, Chah KF, Ononkwo JI, Onyenwe IW, Eze IJ, Iheagwam CN, Njoga EO, Onyema I, Ogbu KI, Mbegbu EC, Nnadozie PN, Ibe EC, Oladimeji KT (2011). Production characteristics, disease prevalence, and herd-health management of pigs in Southeast Nigeria. *J Swine Health Prod.* 19(6): 331–339.

[138]. Odemuyiwa, S.O., Adebayo, I.A., Ammerlaan, W., Ajuwape, A.T.P., Alaka, .O.O., Oyedele, O.I., Soyelu, K.O., Olaleye, D.O., Otesile, E.B., Muller, C.P. (2000). An Outbreak of African swine fever in Nigeria: Virus Isolation and Molecular Characterization of the VP72 Gene of a First Isolate from West Africa *Virus Genes* 20: 139–142

[139]. Odugbemi T. (2011). *Medicinal plant database from Nigeria* (Excel format). Available online at http://www.medicinalplantsinnigeria.com/Plantsdatabase.xls. Accessed on 15th December, 2011.

[140]. Okoth E, Gallardo C, Macharia JM, Baboon K, Lekolol I, Mijele D, Bishop RP. (2009). Participatory assessment of risk factors for African swine fever infection in Western Kenya. *Proceedings of the International Symposium of Veterinary Epidemiology and Economics (ISVEE 12),* Durban, South Africa. Available online at: http://www.sciquest.org.nz/node/67961.

[141]. Olugasa, BO (2007). Serological evidences of African swine fever virus infection in commercial pig herds in south-west Nigeria. *African Journal of Livestock Extension*, 5: 61-66.

[142]. Olugasa, B.O., I.F. Ijagbone. (2007): Pattern of spread of African swine fever in south-western Nigeria, 1997–2005. Vet. Ital. 43: 621–628.

[143]. Onisk D V, Borca M V, Kutish G, Kramer E, Irusta P, RockDL 1994 Passively transferred African swine fever virus antibodies protect swine against lethal infection. *Virology* 198: 350–354.

[144]. Otesile, E.B., Ajuwape, A.T.P., Odemuyiwa, S.O., Akpavie, S.O., Olaifa, A.K., Odaibo, G.N., Olaleye, O.D., Adetosoye, A.I. (2005). Field and Experimental Investigations of an Outbreak of African swine fever in Nigeria. *Rev. Elev. Med. Vet. Pays Trop.* 58, 21–26.

[145]. Owolodun OA, Yakubu B, Haruna ES, Antiabong JF and Shamaki D. (2005). Detection of African swine fever virus in apparently healthy pigs by polymerase chain reaction. *Vom Journal of Veterinary Science* 2: 80–86.

[146]. Owolodun, O.A., B. Yakubu, J.F. Antiabong, O.K. Adefalujo, M.E. Ogedengbe, E.T. Obisakin, and D. Shamaki, 2007: Molecular assessment of African swine fever in North-Central Nigeria. Bull. Anim. Prod. Health Afr. 55, 96–103.

[147]. Owolodun, O.A., Bastos, A.D.S., Antiabong, J.F., Ogedengbe, M.E., Ekong, P.S. and Yakubu B. (2010). Molecular characterisation of African swine fever viruses from Nigeria (2003–2006) recovers multiple virus variants and reaffirms CVR epidemiological utility, Virus Genes, DOI 10.1007/s11262-009-0444-0.

[148]. Penrith M-L. (1998). Control and Eradication of an African swine fever (ASF) Epizootic in Republic of Cape Verde. *Report on the Consultancy conducted between 12 May - 8 June, 1998*. Food and Agricultural Organization of the United Nations Project Symbol: TCP/CVI/8823.

[149]. Penrith, M. L., and Nyakahuma, D. (2000). Recognising African swine fever: A Field Manual. *Animal Health Manual No.9,* FAO, Rome. Food and Agriculture Organization of the United Nations. Rome. Available at: http://www.fao.org/docrep/004/x8060e/x8060e00.htm . Accessed on 6th March, 2010

[150]. Penrith M-L, Thomson G R, Bastos A D S., (2004). African swine fever. In Coetzer J A W, Tustin RC (eds) *Infectious Diseases of Livestock* (2nd edn) Vol. 2. Oxford University Press, Cape Town: 1087–1119.

[151]. Penrith M-L, Thomson G R, Bastos A D S, Phiri O C, Lubisi B A, Botha B, Esterhuysen J, du Plessis E C, Macome F, Pinto F (2004). An investigation into natural resistance to African swine fever in domestic pigs from an endemic area in southern Africa. *Revue Scientifique et Technique, Office International des Épizooties* 23: 665–677

[152]. Penrith, M. L., G. R. Thomson, A. D. Bastos, O. C. Phiri, B. A. Lubisi, E. C. Du Plessis, F. Macome, F. Pinto, B. Botha and J. Esterhuysen. (2004) An investigation into natural resistance to African swine fever in domestic pigs from an endemic area of South Africa. *Revue sci Tech Off Int Epizooties* 23, 665-677.

[153]. Penrith M-L, Lopes Pereira C, Lopes da Silva, M M R, Quembo C, Nhamusso A, Banze J (2007). African swine fever in Mozambique: review, risk factors and considerations for control. *Onderstepoort Journal of Veterinary Research* 74: 149–160

[154]. Penrith, M. L., Guberti, V., Depner, K. and Lubroth, J. (2009). *Preparation of African Swine Fever Contingency Plans*. FAO Animal Production and Health manual No. 8. FAO, Rome, Italy, 71p.

[155]. Penrith M-L, Vosloo, W. (2009). Review of African swine fever:transmission, spread and control. JSAVA, 80(2): 58–62.

[156]. Phologane, S. B., Bastos, A. D. S., Penrith, M-L. (2005). Intra- and inter-genotypic size variation in the central variable region of the *9RL* open reading frame of diverse African swine fever viruses. *Virus Genes* 31, 357–360.

[157]. Pinto, C.J., Urcelay, V.S. (2003). Biosecurity practices on intensive pig production systems in Chile. *Preventive Veterinary Medicine*, 59: 139–145.

[158]. Plowright W and Parker J (1967). The stability of African swine fever virus with particular reference to heat and *p*H inactivation. *Archiv für die Gesamte Virusforschung* **21**: 383–402.

[159]. Plowright, W., Thomson, G.R. and Neser, J.A. (1994). African swine fever. In Coetzer, J.A.W., Thomson, G.R. and Tustin, R.C. (eds). *Infectious diseases of livestock with special reference to southern Africa. 1.* Oxford University Press, Cape Town: pp 568 – 599.

[160]. Polatnick J, Hess W. (1970). Altered thymidine kinase activity in culture cells inoculated with African swine fever virus. *American Journal of Veterinary Research*, 31: 1609–1613

[161]. Polo, J.F. and Sanchez, B.C. (1961). Informe sobre la peste porcina Africana en Espana. Enero-abril 1961 (evolucion, diagnostico y profilaxis). *Bull. Off. int. Epizoot*., 56: 388.

[162]. Posada, D. and Crandall, K.A. (1998). MODELTEST: Testing the model of DNA substitution. *Bioinformatics*, 14: 817–818.

[163]. Pritchard, G., Dennis, I., Waddilove, J. (2005). Biosecurity: reducing disease risks to pig breeding herds. *In Practice*, 27: 230–237.

[164]. Rahim G, Qureshi R, Gulfraz M, Arshad M, Rahim S. (2012). Preliminary phytochemical screening and ethnomedicinal uses of *Teucrium stocksianum* from Makaland Division. *Journal of Medicinal Plant Research*, 6: 704-707.

[165]. Rahimi P, Sohrabi A, Ashrafihelan J, Edalat R, Alamdari M, Mostofi S. (2010). Emergence of African swine fever virus, Northwestern Iran. *Emerging Infectious Diseases* 16(12):1946-1948

[166]. Randriamparany, T., Grenier, A., Tourette, I., Maharavo-Rahantamala, C.Y., Rousset, D., Lancelot, R. (2005). Epidemiological situation of African swine fever in Lake Alaotra Region (Madagascar) and possible consequences on the organization of disease control and surveillance. *Revue Elevage Medicine Veterinaire. Pays Tropicaux.* 58, 15–20.

[167]. Rendleman CM, Spinelli FJ (1999). The costs and benefits of animal disease prevention: the case of African swine fever in the US. *Environmental Impact Assessment Review*, 19: 405-426.

[168]. Ribbens S, Dewulf J, Koenen F, Mintiens K, de Sadeleer L, de Kruif A, Maes D. (2008). A survey of biosecurity practices in Belgian pig herds. *Preventive Veterinary Medicine*, 83: 228-241.

[169]. Rivas, A.L., Chowell, G., Schwager, S.J., Fasina, F.O., Hoogesteijn, A.L., Smith, S.D., Bisschop, S.P.R., Anderson, K.L. (2010). Lessons from Nigeria: the role of roads in the geo-temporal progression of the avian influenza (H5N1). *Epidemiology and Infection*, 138, 192–198.

[170]. Rodriguez, J. M., Yanez, R. J., Pan, R., Rodriguez, J. F., Salas, M. L. and Vinuela, E. (1994). Multigene families in African swine fever virus: Family 505. *Journal of Virology,* 68 (4): 2746-2751.

[171]. Roger F, Ratovonjato J, Vola P and Uilenberg G. (2001). Ornithodoros porcinus ticks, bushpigs, and African swine fever in Madagascar. *Experimental and Applied Acarology,* 25:263-269.

[172]. Ronquist, F. and Huelsenbeck, J. P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

[173]. Rowlands, R.J., Michaud, V., Heath, L., Hutchings, G., Oura, C., Vosloo, W., Dwarka, R., Onashvili, T., Albina, E. and Dixon, L.K. (2008). African swine fever virus isolate,

Georgia, 2007. *Emerging Infectious Diseases*, 14 (12): 1870-1874. DOI: 10.3201/eid1412.080591.

[174]. Rychlik, W. (1993). Selection of primers for the polymerase chain reaction. In: *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*. Humana Press Inc., Totowa,. Pp 31-39.

[175]. Saka, JO, Adesehinwa AOK, Ajala, MK. (2010). Incidence of African swine fever (ASF) disease and its associated implications on pig production in Lagos state, Nigeria. *Bulgarian Journal of Agricultural Science,* 16 (1): 80-90.

[176]. Samui KL, Nambota AM, Mweene AS, Onuma M. (1996). African swine fever in Zambia: Potential financial and production consequences for the commercial sector. *Japanese Journal of Veterinary Research,* 44(2): 119-124.

[177]. Sanchez-Vizcaıno, J. M., 2006: African swine fever. In: Straw, B. E., J. J. Zimmerman, S. D'Allaire, and D. J. Taylor (eds), Diseases of Swine, 9th edn, pp. 291–298. Blackwell Publishing Professional, Ames, Iowa.

[178]. Sanchez-Vizcaıno, J. M., Mur, L., Martinez-Lopez, B., (2012). African swine fever: An epidemiological update. *Transboundary and Emerging Diseases*, 59 (Suppl. 1): 1-9.

[179]. Schwikkard S, van Heerden FR. (2002). Antimalarial activity of plant metabolites. *Natural Product Reports*, 19: 675-692.

[180]. Shimadzu Corporation. *GC-MS QP 2010 Plus brochure*. (2012). Available online at http://www.ssi.shimadzu.com/products/literature/Mass_Spec/GCMS-QP2010_Plus_F.pdf, Accessed on 11th June, 2012.

[181]. Standing Committee of Food Chain and Animal Health (SCFCAH) (2011). African swine fever in Sardinia: epidemiological situation 04 November, 2011. http://ec.europa.eu/food/committees/regulatory/scfcah/animal_health/presentations/08091111_asf_sardinia_italy.pdf. Accessed on 4th March, 2012.

[182]. Stalder, K., L. Engblom, J. Mabry. (2010). Benchmarking sow lifetime productivity. Available at http://www.benchmark.farms.com/Benchmarking_Sow_Lifetime_Productivity.html. Accessed on August 22, 2010.

[183]. Stärk, K., Pfeiffer, D., Morris, R. (1998). Risk factors for respiratory diseases in New Zealand pig herds. *New Zealand Veterinary Journal*, 46, 3–10.

[184]. Silva O, Barbosa S, Diniz A, Valdeira ML, Gomes E. (1997). Plant extracts antiviral activity against Herpes simplex virus type 1 and African swine fever. *Pharmaceutical Biology,* 35: 12-16.

[185]. Simmons MP, Ochoterena H. (2000). Gaps as character in sequence-based phylogenetic analyses. *Systematic Biology*, 49: 369-381.

[186]. Skinner JD, Smithers RHN. (1990). *The Mammals of the Southern African Sub-region*. 2nd ed. University of Pretoria Press, Pretoria.

[187]. Sofowora A. (1993). *Medicinal Plants and Traditional Medicine in Africa*, John Wiley and Sons Ltd., 150-153.

[188]. Sumption, K. J., Hutchings, G. H., Wilkinson P. J. and Dixon, L. K. (1990). Variable regions on the genome of Malawi isolates of African swine fever virus. *Journal of General Virology* 71, 2331–2340.

[189]. Swofford DL. (2002). *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods).* Version 4.0b 10. MA, Sinauer Associates, Inc., USA, 2002.

[190]. Tambi, E.N., Maina, O.W., Mukhebi, A.W., Randolph, T.F. (1999). Economic impact assessment of rinderpest control in Africa. *Rev. Sci. Tech. Off. Int. Epizoot.*, 18: 458–477.

[191]. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*, 28: 2731-2739.

[192]. Taylor, W.P., Best, J.R. and Colquhoun, I.R. (1977). Absence of African swine fever from Nigerian warthogs. Bulletin of Animal Health and Production in Africa 25: 196-203.

[193]. Thomas, D. W. and Gereau, R. E. (1993). Ancistrocladus korupensis (Ancistrocladaceae): A new species of Liana from Cameroon. *Novon*, 3: 494-498.

[194]. Thomson G.R. (1985). The epidemiology of African swine fever: the role of free-living hosts in Africa. *Onderstepoort Journal of Veterinary Research*, 52 (3), 201-209.

[195]. Tisdell, C.A., Harrison, S.R., Ramsay, G.C. (1999). The economic impacts of endemic diseases and disease control programmes. *Rev. Sci. Tech. Off. Int. Epizoot.*, 18: 380–398.

[196]. Trease GE, Evans WC. (1996). *A Textbook of Pharmacognosy*. 1st Ed., Williams and Wilkins Company, Baltimore, Maryland.

[197]. Tulman, E.R., Delhon G.A., Ku B.K., Rock D.L.(2009). African swine fever virus. *Curr Top Microbiol Immunol.* 328:43-87.

[198]. USDA-ERS. (2009). ERS/USDA Briefing Room-Hogs: Trade. Available at http://www.ers.usda.gov/Briefing/Hogs/trade.htm . Accessed on 7th March, 2010.

[199]. Vaillancourt, J.P., Carver, D.K. (1998). Biosecurity: perception is not reality. *Poultry Dig*. 57: 28-36.

[200]. Valadão F G (1969). Notas sobre alguns ensaios realizadosemporcos sobreviventes de peste suína africana. *Anais dos Serviços Veterinárias de Moçambique* No 12/14, 1964–1966 (1969): 95–100

[201]. Vial, L., Wieland, B., Jori, F., Etter, E., Dixon, L. and Roger, F. (2007). African swine fever virus DNA in soft ticks, Senegal. *Emerging Infectious Diseases* 13 (12): 1928-1931. Also available at: www.cdc.gov/eid.

[202]. Vanden Berghe DA, Vlietinck AJ, Vanhoof L. (1986). Plant products as potential antiviral agents. *Bulletin Institute Pasteur*, 84: 101-147.

[203]. Vercammen, P., A. H. W. Seydack, and W. L. R. Oliver. 1993. The bush pigs (*Potamochoerus porcus* and *P. larvatus*). *In* Pigs, Peccaries and Hippos: Status Survey and Action Plan. *Edited by* W. L. R. Oliver. Gland, Switzerland: IUCN. pp. 93-101. Available online at http://iucn.org/themes/ssc/sgs/pphsg/Contents.htm and (http://www.ultimateungulate.com/Artiodactyla/Potamochoerus_porcus.html ; http://www.ultimateungulate.com/Artiodactyla/Potamochoerus_larvatus.html ; http://www.ultimateungulate.com/Artiodactyla/Hylochoerus_meinertzhageni.html ; http://en.wikipedia.org/wiki/Warthog)

[204]. Wentland MP, Bailey DM, Cornett JB, Dobson RA, Powles RG, Wagner RB. (1984). Novel amino-substituted 3-quinolinecarboxylic acid antibacterial agents: synthesis and structure-activity relationship. *Journal of Medicinal Chemistry*, 27: 1103-1108.

[205]. Wesley, R.D., Tuthill, A.E., (1984). Genome relatedness among African swine fever virus field isolates by restriction endonuclease analysis. *Preventive Veterinary Medicine* 2: 53–62.

[206]. Wilkinson, P. J. (1989). African swine fever virus. In: M. B. Pensaert, (Ed), *Virus Infections of Porcines*, Elsevier, Amsterdam, pp. 17–37.

[207]. World Organisation for Animal Health (OIE) (2008). African swine fever. In: *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. Web edition. Available at http://www.oie.int/eng/normes/mmanual/2008/pdf/2.08.01_ASF.pdf . Accessed on 20th January, 2009.

[208]. World Organisation of Animal Health (OIE). (2010). WAHID Interface: Animal Health Information (Weekly Disease Information). Available at http://www.oie.int/wahis/public.php?page=weekly_report_index . Accessed on 11 March, 2012.

[209]. World Organisation of Animal Health (OIE) (2011). Handistatus II. Multiannual ASF Disease incidence (new outbreaks). Available at: http://web.oie.int/hs2/sit_mald_freq_pl.asp?c_cont=1andc_mald=13. Accessed on 29 December, 2011.

[210]. World Organisation of Animal Health (OIE) (2012). WAHID Interface: Animal Health Information. Available at http://web.oie.int/wahis/public.php?page=weekly_report_index

[211]. World Organization for Animal Health (OIE). Early detection and contingency plans for African swine fever. *Recommendation 2 of the 24th Conference of the OIE Regional Commission for Europe*, Astana, Kazakhstan, 20-24 September, 2011. Available at: http://www.oie.int/doc/ged/D10785.PDF. Accessed on 11 March, 2012.

[212]. World Organization for Animal Health (OIE): WAHID Interface: Animal Health Information. Weekly Summary. Available at: http://web.oie.int/wahis/public.php?page=weekly_report_index. Accessed on 11 March, 2012.

[213]. Woolridge M, Hartnett E, Cox A, Seaman M. (2006). Quantitative risk assessment case study: smuggled meats as disease vectors. *Rev. sci. tech. Off. int. Epiz.*, 25 (1): 105-117.

[214]. Yanez RJ, Rodriguez JM, Rodriguez JF, Salas ML, Vinuela E. (1993). African swine fever virus thymidylate kinase gene: sequence and transcriptional mapping. *Journal of General Virology*, 74: 1633-1638.

[215]. Yanez, R. J., Rodriguez, J. M., Nogal, M. L., Yuste, L., Enriquez, C., Rodriguez, J. F. and Vinuela, E. (1995). Analysis of the complete nucleotide sequence of African swine fever virus. *Virology,* 208:249-278.

[216]. Ying-Wang C, Huang S-C, Zhang Y, Lai Z.-R, Kung S-H, Chang Y-S, Lin C-W. (2012). Antiviral ability of *Kalanchoe gracilis* leaf extract against Enterovirus 71 and Coxsackievirus A16. *Evidence-Based Complementary and Alternative Medicine*, Article ID 503165; doi: 10.1155/2012/503165.

[217]. Zsak, L., Lu, Z., Kutish, G.F., Neilan, J.G., Rock, D.L. (1996). An African swine fever virus virulence-associated gene *NL-S* with similarity to the Herpes Simplex virus *ICP34.5* gene. J Virol., 70(12):8865-8871.

[218]. Zsak, L., Caler, E., Lu, Z., Kutish, G.F., Neilan, J.G., Rock, D.L. (1996). A Nonessential African swine fever virus gene *UK* is a significant virulence determinant in domestic swine. J Virol., 72(2):1028-1032.

Appendix A: Multiannual ASF disease incidence (Africa, New Outbreaks, 1996-2004)

Country/Territory	1996	1997	1998	1999	2000	2001	2002	2003	2004
Algeria	0000	0000	0000	0000	0000	0000	0000	0000	0000
Angola	+	+	+	+	+	+	+	+	+
Benin		+()	+		+	+	+	+	+
Botswana	(11/1987)	(11/1987)	(11/1987)	+	(06/1999)	(06/1999)	(06/1999)	(06/1999)	(06/1999)
Burkina Faso	0000	0000	0000	0000	0000	0000	0000	+	+
Burundi						+		+	+
Cameroon	+	+()	+()	+()	+()	+()	+()	+()	+()
Cape Verde	+	+	+	+			?	?	...
Central African Republic	0000						0000	0000	0000
Chad	0000	0000	0000	0000	0000	0000	0000	0000	0000
Comoros		...	0000						
Congo (Dem. Rep. of the)			-			+	+	+	+
Congo (Rep. of the)					+	+	?	+	
Côte d'Ivoire	+	(1996)	(12/1996)	(12/1996)	(12/1996)	(12/1996)	(12/1996)	(12/1996)	(12/1996)
Djibouti							0000	0000	0000
Egypt	0000	0000	0000	0000	0000	0000	0000	0000	0000
Eritrea	...	...		-	-	-	-	-	-
Ethiopia	(1993)	(1993)	(1993)	(1993)	(1993)	(1993)	(1993)	(1993)	(1993)
Gabon		?				-	-	-	
Gambia	...	+							
Ghana	0000		0000	+	+	(02/2000)	+	+	+
Guinea	0000		-	-	-	-	-	-	-
Guinea-Bissau							-	?	+
Kenya	(11/1994)	(11/1994)	(11/1994)	(11/1994)	(11/1994)	+	(08/2001)	(08/2001)	(08/2001)
Lesotho	0000	0000	0000	0000	0000	0000	0000	0000	
Libya	0000	0000	0000	0000	0000	0000	0000	0000	0000
Madagascar	0000	0000	+	+	+	+	+	+	+
Malawi	+	+	+	+	+	+	+	+	+
Mali	-	-		-	-	-	-	-	-
Mauritania		...				-	-		
Mauritius	0000	0000	0000	0000	0000	0000	0000	0000	0000
Morocco	0000	0000	0000	0000	0000	0000	0000	0000	0000
Mozambique	+()	+()	+()	+	+	+	+	+	+
Namibia	(10/1995)	+	+	(04/1998)	(04/1998)	+	(11/2001)	(11/2001)	+
Niger	-	-	-	-	-	-	-		
Nigeria		+	+	+	+	+	+	+	+
Reunion (France)	0000	0000	0000	0000	0000	0000	0000	0000	0000
Rwanda							+	+	+
Sao Tome and Principe				(1992)	(1992)	(1992)	(1992)	(1992)	(1992)
Senegal	+	+	+	+	(07/1999)	+	+	+	+
Seychelles	0000	0000	0000		0000		0000		-
Somalia					0000		-		-
South Africa	+()	+()	+()	(12/199	(12/199	+()	+()	+()	+()

				8)	8)				
Sudan	0000	0000	0000	0000	0000	0000	0000	0000	0000
Swaziland	0000	0000	0000	0000	0000	0000	0000	0000	0000
Tanzania	...	-	-	-	-	+	+	+	+
Togo		+()	+		+	+	+	+	+
Tunisia	0000	0000	0000	0000	0000	0000	0000	0000	0000
Uganda	+	+	+	+	+	+	+	+	+
Zambia	(12/1995)	+()	+()	+	+	+	+		+
Zimbabwe	(03/1992)	(03/1992)	(03/1992)	(03/1992)	(03/1992)	(03/1992)	(03/1992)	(03/1992)	(03/1992)

Codes

0000	Disease never reported
-	Disease not reported (date of last outbreak not known)
(month/year)	Date of the last reported occurrence of the disease in previous years
?	Disease suspected but presence not confirmed
+	Reported present or known to be present
+?	Serological evidence and/or isolation of the causal agent, but no clinical signs of disease
( )	Disease limited to specific zones
...	No information available

OIE, 2011. Handistatus: Multiannual ASF Disease Incidence (1996-2004).

Appendix B: Global picture/Disease Timelines for African swine fever, 2005-2011

Key to colours

	There is no information available on this disease
	Never reported
	Disease not reported during this report period
	Disease suspected but not confirmed
	Confirmed infection but no clinical disease
	Confirmed clinical infection
	Confirmed infection but limited to certain zones
When different animal health statuses between domestic and wild animal population are provided the box is split in two: • The upper part indicates the situation in domestic animals • The lower part indicates the situation in wild animals	
N	Note
NA	Not Applicable

Start year	2005	End year	2012	OK

Country	Status for six month periods														
	2005		2006		2007		2008		2009		2010		2011		2012
	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun	Jul-Dec	Jan-Jun
Afghanistan															
Albania															
Algeria															
Andorra															
Angola															
Argentina															
Armenia															
Aruba															
Australia															
Austria															
Azerbaijan															
Bahrain															
Bangladesh															
Barbados															
Belarus															
Belgium															
Belize															
Benin															
Bhutan															
Bolivia															
Bosnia and Herzegovina															
Botswana															
Brazil															
Brunei Darussalam															

Bulgaria															
Burkina Faso															
Burundi															
Cambodia															
Cameroon															
Canada															
Cape Verde															
Cayman Islands															
Central African Republic															
Chad															
Chile															
China (People's Rep. of)															
Chinese Taipei															
Colombia															
Comoros															
Congo (Dem. Rep. of the)															
Congo (Rep. of the)															
Costa Rica															
Cote D'Ivoire															
Croatia															
Cuba															
Cyprus															
Czech Republic															
Denmark															
Djibouti															
Dominica															
Dominican Republic															
Ecuador															
Egypt															
El Salvador															
Equatorial Guinea															
Eritrea															
Estonia															
Ethiopia															
Fiji															
Finland															
Former Yug. Rep. of Macedonia															
France															
French Guiana															
French Polynesia															
Gabon															
Gambia															
Georgia															
Germany															
Ghana															
Greece															
Greenland															

Grenada																
Guadeloupe (France)																
Guatemala																
Guinea																
Guinea-Bissau																
Guyana																
Haiti																
Honduras																
Hong Kong (SAR - PRC)																
Hungary																
Iceland																
India																
Indonesia																
Iran																
Iraq																
Ireland																
Israel																
Italy																
Jamaica																
Japan																
Jordan																
Kazakhstan																
Kenya																
Kiribati																
Korea (Dem. People's Rep.)																
Korea (Rep. of)																
Kuwait																
Kyrgyzstan																
Laos																
Latvia																
Lebanon																
Lesotho																
Libya																
Liechtenstein																
Lithuania																
Luxembourg																
Madagascar																
Malawi																
Malaysia																
Maldives																
Mali																
Malta																
Martinique (France)																
Mauritania																
Mauritius																
Mexico																
Micronesia (Federated States)																
Moldavia																
Mongolia																
Montenegro																
Morocco																

Mozambique																
Myanmar																
Namibia																
Nepal																
Netherlands																
New Caledonia																
New Zealand																
Nicaragua																
Niger																
Nigeria																
Norway																
Oman																
Pakistan																
Palestinian Auton. Territories																
Panama																
Papua New Guinea																
Paraguay																
Peru																
Philippines																
Poland																
Portugal																
Qatar																
Reunion (France)																
Romania																
Russia																
Rwanda																
Samoa																
San Marino																
Saudi Arabia																
Senegal																
Serbia																
Serbia and Montenegro																
Seychelles																
Sierra Leone																
Singapore																
Slovakia																
Slovenia																
Somalia																
South Africa																
Spain																
Sri Lanka																
St. Kitts and Nevis																
St. Vincent and the Grenadines																
Sudan																
Suriname																
Swaziland																
Sweden																
Switzerland																
Syria																
Tajikistan																
Tanzania																

Thailand																
Togo																
Tonga																
Trinidad and Tobago																
Tunisia																
Turkey																
Turkmenistan																
Tuvalu																
Uganda																
Ukraine																
United Arab Emirates																
United Kingdom																
United States of America																
Uruguay																
Uzbekistan																
Vanuatu																
Venezuela																
Vietnam																
Wallis and Futuna Islands																
Yemen																
Zambia																
Zimbabwe																

Appendix C: Questionnaire Survey on Impacts and Epidemiology of African swine fever in Nigeria

This questionnaire is collated and being conducted as part of an on-going Doctor of Veterinary Medicine Project. It is a non-profit/non-commercial research meant for the public good. The privacy of all participants will be strictly ensured and any information provided will be used only for the purpose of this research.

PART I

QUESTIONNAIRES FOR THE PIGGERY OPERATION

SECTION A: GENERAL

s/no.		
1	State	
2	Location	
4	Name (optional)	
4	Marital Status	
5	Age	Below 20……. 20-30………. 31-40………. 41-50………. Above 50……..
6	Occupation (Farmer)	
7	Education level	Number of years of school………………

SECTION B: FARM OPERATION

Is pig farming your main occupation? Yes……………….No……………

If yes, do you have a secondary occupation? Yes…………………No………

If no, what is your main occupation? ……………………………………………………

Averagely, what % of your time is dedicated to pig farming? ……………………….

Where did you raise money to start pig farming? Self…………, Loan…………, Others (state)………

Do you still have loans to pay back? ……………………………………………………

How do you dispose/sell your pig products? ………………………………..

Do farm-gate buyers collect pig/pig product from your farm? Yes….. No…….

Do you have pig abattoir in your premises? ……………………………………………

Where do they take the pig/pig product to? ………………………………………………………......

What types/breed of pigs do you keep?

Types of Pigs	Number	Age	Source of pig

Did African swine fever **affect** your farm in any way? Yes……. No………

If yes, how (mark as many as applicable)?

Lose pigs	Lose income source	Reduction in income	Spend more on disease prevention	Cannot sell products	Others(state)

Did African swine fever **infect** (cause disease) your farm? Yes…….No……….

(This section can be skipped for uninfected farms)

When was your farm infected? ....

To whom did you report? ……………………………………………………………………..

How long does it take you between disease infection and reporting? ……………………….

How easily can you report African swine fever outbreak?

Easy	Not easy	Very difficult

Are you doing any other job now if you lose all your pigs? Yes…….. No……

………

……………………………………………………………………………………………………..

Have you gone back to piggery farming? Yes…. No……Later…. Never…...

Were you paid any compensation? Yes………………. No……………………..

Are you happy with the compensation paid? Yes………… No……………

Rate the following in order of their importance to you ?

More compensation	Information/counselling	Re stocking	Re financing	Others (state)

What do you think is responsible for infection in your farm (infected farms only)?

………

………

Did you visit any infected farm just before the outbreak? Yes…..No……Not sure……….

If yes, how long? ………………………….

Did you receive visitors from infected farms just before the outbreak? Yes….No….Not sure…..

If yes, how long? ……………………….

How did you sell/dispose off your product during the outbreak?

Rapid slaughter and sale in open market	Destroy and bury/burn	Dispose off in the refuse dump	Slaughter and eat/sell	Government officials handle it

Did you purchase live pigs **before your farm was infected**? Yes…… No……….

Did you purchase pig products **before your farm was infected**? Yes…. No…..?

What quantity? …………………………………………….

(This section apply to all farms)

SECTION C: EPIDEMIOLOGY

Do you visit other people's farm? Yes…… No………

Do you have infected farms in your immediate neighbourhood? Yes…… No……Not sure…..…

Do you keep other categories of animals? Yes……… No………… If yes, list

How do you dispose your farm litter and other waste materials from the farm?

Burn/bury	Sell as fertilizer	Dump in refuse site	Spread in farm site	Other (state)

How do you dispose your pig intestines and other slaughter waste materials following abattoir procedure ?

Burn/bury	Sell for consumption	Dump in refuse site	Dispose indiscriminately	Other (state)

Do wild birds have access to such intestinal content? Yes……… No …………..

Do these wild birds visit your farm? Yes………., No…………………

Do you compound your animal feed or you buy finished (ready-made) feed? ……………

Do you see engorged ticks on your pigs? Yes ……………, No………………….

Do you borrow farm equipment? Yes………… No…………, If yes, what? ....

....

What is the water source for your farm? ……………. Do you share this source with other farms? Yes……… No………….

Do you have abattoir debris around your farm? Yes………… No…………..

Do you have problem with rat in your farm? Yes………… No…………

PART II

BIOSECURITY, MANAGEMENT PRACTICES AND COSTING FOR THE OPERATIONS.

1) Which of the biosecurity measures tabulated below is practiced or present in the farm? Tick all observed measures. If **"no"**, which one are you willing to adopt?

s/no.	BIOSECURITY MEASURES	Yes	No	Practicability	Willingness to adopt the measure	Associated costs per annum (Naira)
1	Restricted access to all visitors					
2	Fence around premises					
3	Gate at entrance					
4	Wire mesh window					
5	Foot dips for disinfection before the house					
6	Record keeping					
7	Food and water control					
8	Terminal (End of operation) cleaning					
9	Routine(regular) cleaning					
10	Safe disposal of faeces and dead pigs (protected away from other animal and insect)					
11	Quarantine newly purchased pigs for at least 10 days					
12	Regular cleaning and disinfection of feeders and drinkers					
13	Sufficient feeding and watering space available for all pigs					
14	Sufficient space for each pig (No overcrowding)					
15	Remove manure and litter routinely.					
16	Usage of Disinfectant after cleaning					
17	Lock for each pen					
18	Assess Health status of pigs coming in					
19	Do not mix different ages					
20	Do not mix different species					
21	All-in all-out production					
22	Hand sanitizer, gloves and washing					
23	Going from young to older pigs					
24	Change clothing when going in/out					
25	Separate sick pigs					
26	Consult with a veterinarian in case of sick pigs					
27	Change rubber boots/slippers					
28	Wash/disinfect equipment and tools					
29	Downtime $\geq$ 2 weeks					
30	Pest control (rodents & insects)					
31	Prompt sick/ dead bird disposal from the farm					
32	Change solution in foot pans regularly					
33	Auditing: incentives, education, adherence (encourage assistants to adhere to biosecurity)					

Thank you for your time.

Appendix D1-3 (PDF files)

RESULTS OF ANALYSIS OF *ANCISTROCLADUS KORUPENSIS* BASED ON GAS CHROMATOGRAPH-MASS SPECTROMETRIC ASSAYS

D1: WHOLE PLANT (STEM BARKS, LEAVES AND ROOTS)

D2: LEAVES

D3: STEM BARKS

Uploaded online as supplementary pdf files only.

Appendix F (Password protected-loaded as a separate file online).

STANDARD OPERATING PROCEDURE FOR THE DETECTION OF AFRICAN SWINE FEVER VIRUS (ASFV) BY REAL TIME POLYMERASE CHAIN REACTION (PCR)

Copyright: CISA-INIA, Valdeolmos, Spain.

APPENDIX E: TRANSLATED AMINO ACIDS OF *Thymidine Kinase* genes of ASF VIRUSES USED IN THE STUDY

```
AF504895_LIS_57                  MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
Dakar_59                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF504891_LIS_60                  MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
Angola_70                        MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
m63119_TK_ref_E70(MS44)          MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
Z21490_BA71(V)                   MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF504892_MALTA_78                MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
NAM_1_80                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
LIV_5_40                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF504896_CAM_4_85                MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
CAM_1_86                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
DRC_624_89                       MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
AF504899_SPEC_208AB              MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
SPEC_209                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF504890_NUR_1_90                MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF504902_VICT_90_1               MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
IC_2_96                          MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
IC_3_96                          MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF504893_NIG_1_99                MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF504897_MAD_1_98                MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
BOT_1_99                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
SPEC_140                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
AF504901_RSA_1_99W               MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLEHLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKHCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
AF504900_MOZ_94_1                MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VAFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
ZIM_92_1                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF504898_SPEC_120                MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
SPEC_125                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
RSA_2_96                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKI IESTQLSDVG SLIDIHAVVI DEAHFFDDLI  [100]
AF504894_RSA_3_96                MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKI IESTQLSDVG SLIDIHAVVI DEAHFFDDLI  [100]
LILLIE                           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKI IESTQLSDVG SLIDIHAVVI DEAHFFDDLI  [100]
RSA_1_96_AB                      MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKHCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
SPEC_254_AB                      MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
RHO_61_1                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLQSKQCEI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
AF504903_MALAWI_1978             MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLQSKQCEI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
MOZ_A_98                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLQSKQCEI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
KAB_6/2                          MNIIKKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLQSKQCKI IESTQLSDVG FLTDIHVVVI DEAHFFDDLI  [100]
MZI_92_1                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLQSKQCEI IESTQLSDVG SLTDIHAVVI DEAHFFNDLI  [100]
SUM_14_11                        MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKL IESTQLSDVG SLTDIHAVVI DEAHFFDDLM  [100]
NYA_1_2AB                        MNIIKKLKPG TISLVLGPMF AGKTTFLIYC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
TAN_1_01                         MNIIKKLKPG TISLVLGPMF AGKTTFLIHC IYLFERLEKK VVFIKSTKNT RDKTVKTHSG IQLQSKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLM  [100]
TAN_2003_1                       MNIIKKLKPG TISLVLGPMF AGKTTFLIHC IYLFERLEKK VVFIKSTKNT RDKTVKTHSG IQLQSKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLM  [100]
UGA_1_95                         MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESTQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
AF504905_HINDE_II                MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESTQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
AF504904_KWH_12                  MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESAQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
AF504906_BUR_84_1                MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESAQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
AF504907_UGA_95_3                MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESAQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
AF270708_MOZ/60                  MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VAFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
MAD/62                           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
24823                            MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
AY351515_ZAIRE_82                MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
FJ455837_Mkuzi_GR21-23           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
FJ455836_Mkuzi_GR21-11           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
FJ455838_Mkuzi_GR22-6            MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
AF302810_Dominican_Republic      MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF301543_MALTA                   MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF504881_SWA_Baby_warthog19      MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF301544_CAM/82                  MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
BUR_84/2                         MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESAQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
SPEC_89                          MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF449467_HOL/86                  MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
ZAW_88/1                         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLQSKQCEI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
```

```
OURT_88/2                     MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF3012811_OURT_88/1           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF511452_CAM_89/1             MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
DQ250114_SPEC_205             MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF270712_MOZ_94/8             MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VAFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
AF270710_SPEC_265             MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VAFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
RSA/P/4/95/+                  MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKI IESTQLSDVG SLIDIHAVVI DEAHFFDDLI  [100]
DQ250123_RSA/P/1/95/+         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
JX403652_BEN_97/5             MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF302816_BEN_97               MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
JX403650_BEN_97/2             MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
JX403651_BEN_97/3             MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF302816_BEN/P/1/97/+         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AY972164_BEN_97/4             MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF302817_NIG/P/1/98/+         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AY972161_NIG/P/2/98/+         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
NIG/P/3/98/+                  MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF449478_GAM/1/00             MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AF504888_GHA/P/1/00/+         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
JX524215_KEN_2001/2           MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESTQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
JX403676_KEN_2001/6           MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESTQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
JX403675_KEN_2001/5           MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESTQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
AY494550_TAN/2001/3           MNIIKKLKPG TISLVLGPMF AGKTTFLIHC IYLFERLEKK VVFIKSTKNT RDKTVKTHSG IQLQSKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLM  [100]
JX403672_KEN_2001/1           MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESTQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
RSA_2001/2                    MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
JX403669_CAM_2002/4           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
RSA2002/1                     MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRSKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
JX403666_CAM_2002/1           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
JX403668_CAM_2002/3           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
JX430662_GHA_2002/1           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
JX403656_CAM_2002/5           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
JX403663_GHA_2002/2           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AY494553_MAL_2002/1           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCEI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
JX403667_CAM_2002/2           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AY351564_UGA_2003/1           MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESTQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
JX403661_B_Faso_2003/1        MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
NAM_2004/3                    MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
JX403637_NAM_2004/1           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
JX403645_RSA_2004/3           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVI DEAHFFDDLI  [100]
Kenya05_Tk1                   MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INTLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESTQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
NIG_01/1                      MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
Kenya06_Bus                   MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESTQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
1546C/06                      MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112582 NIG_08/PLJs16        MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112583 NIG_08/PLJs42        MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112584 NIG_08/PLJs43        MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
JX310054_B_Faso_2007/1        MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
FJ528594_Mauritius_2007/1     MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112585 NIG_08/BNGb2         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112586 NIG_08/BNGb4         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112587 NIG_08/BNGb9         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112588 NIG_08/BNGb24        MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112589 NIG_08/BNMk42        MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112590 NIG_08/LAOk1         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112591 NIG_08/LAOk2         MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112592 NIG_08/NW6           MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112593 NIG_08/NW10          MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
KC112594 NIG_08/NW12          MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
Vic/UGA                       MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESAQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
AM712240_OURT_88_3            MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AY261360_Kenya1950            MSLIRKLKPG TISLVLGPMF AGKTTFLIHC INMLERLEKK VVFIKSTKNT RDKTIQTHSG IQLRPNQCKI IESAQLSDVG SLTDTHAVVI DEAHFFDDLI  [100]
FN557520_E75_Spain            MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
AM712239_Benin_97_1_pathogenic MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
FR682468_Georgia_2007_1       MNIIRKLKPG TISLVLGPMF AGKTTFLIHC IYMLERLEKK VVFIKSTKNT RDKTIKTHSG IQLRPKQCKI IESTQLSDVG SLTDIHAVVV DEAHFFDDLI  [100]
```

Isolate	Sequence	Length
AF504895_LIS_57	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
Dakar_59	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504891_LIS_60	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
Angola_70	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
m63119_TK_ref_E70(MS44)	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
Z21490_BA71(V)	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504892_MALTA_78	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
NAM_1_80	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
LIV_5_40	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504896_CAM_4_85	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
CAM_1_86	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
DRC_624_89	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504899_SPEC_208AB	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
SPEC_209	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504890_NUR_1_90	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504902_VICT_90_1	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
IC_2_96	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
IC_3_96	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504893_NIG_1_99	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504897_MAD_1_98	TCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
BOT_1_99	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
SPEC_140	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504901_RSA_1_99W	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504900_MOZ_94_1	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKALILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
ZIM_92_1	TCRTWADEEK IIILAGLNAS FEQKMFPSIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504898_SPEC_120	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
SPEC_125	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
RSA_2_96	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMRCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504894_RSA_3_96	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMRCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
LILLIE	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMRCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
RSA_1_96_AB	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
SPEC_254_AB	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
RHO_61_1	KCRAWADEEK IIILAGLNAS FEQKMFQPIV HIFPYCSWIK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNKCIKP LQPIKY*	[197]
AF504903_MALAWI_1978	KCRAWADEEK IIILAGLNAS FEQKMFQPIV HIFPYCSWIK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKKQMY*	[188]
MOZ_A_98	KCRAWADEEK IIILAGLNAS FEQKMFQPIV HIFPYCSWIK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNKCIKP LQPIKY*	[197]
KAB_6/2	KCRAWADEEK IIILAGLNAS FEQKMFQPIV HIFPYCSWIK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN KCLKNKCIKP LQPIKY*	[197]
MZI_92_1	KCRAWADEEK IIILAGLNAS FEQKMFQPIV HIFPYCSWIK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKK*	[185]
SUM_14_11	KCRTWADEEK IIILAGLNAS FEQKMFPSIV HIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN ACLKK*	[185]
NYA_1_2AB	KCRAWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSKLYVTCCN NCLKK*	[185]
TAN_1_01	KCRAWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKK*	[185]
TAN_2003_1	KCRAWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKK*	[185]
UGA_1_95	RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKSLILAG GSELYVTCCN NCLKK*	[185]
AF504905_HINDE_II	RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYITCCN NCLKK*	[185]
AF504904_KWH_12	RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYITCCN NCLKK*	[185]
AF504906_BUR_84_1	RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYITCCN NCLKK*	[185]
AF504907_UGA_95_3	RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYITCCN NCLKK*	[185]
AF270708_MOZ/60	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKALILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
MAD/62	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
24823	KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AY351515_ZAIRE_82	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
FJ455837_Mkuzi_GR21-23	KCRTWADEEK IIILAGLNAS FEQKMFPSIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKKYIY*	[188]
FJ455836_Mkuzi_GR21-11	KCRTWADEEK IIILAGLNAS FEQKMFPSIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKKYIY*	[188]
FJ455838_Mkuzi_GR22-6	KCRTWADEEK IIILAGLNAS FEQKMFPSIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKKYIY*	[188]
AF302810_Dominican_Republic	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF301543_MALTA	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF504881_SWA_Baby_warthog19	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF301544_CAM/82	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
BUR_84/2	RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYITCCN NCLKNKFIK*	[189]
SPEC_89	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF449467_HOL/86	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
ZAW_88/1	KCRAWADEEK IIILAGLNAS FEQKMFQPIV HIFPYCSWIK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKKQMY*	[188]
OURT_88/2	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF3012811_OURT_88/1	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]
AF511452_CAM_89/1	KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY*	[197]

```
DQ250114_SPEC_205             KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AF270712_MOZ_94/8             KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKALILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AF270710_SPEC_265             KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKALILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
RSA/P/4/95/+                  KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMRCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKSTFIKQ LQPIKY* [197]
DQ250123_RSA/P/1/95/+         KCRTWADEEK IIILAGLNAS FEQKMFPSIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKKYIY*          [188]
JX403652_BEN_97/5             KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AF302816_BEN_97               KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
JX403650_BEN_97/2             KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
JX403651_BEN_97/3             KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AF302816_BEN/P/1/97/+         KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AY972164_BEN_97/4             KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AF302817_NIG/P/1/98/+         KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AY972161_NIG/P/2/98/+         KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
NIG/P/3/98/+                  KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AF449478_GAM/1/00             KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AF504888_GHA/P/1/00/+         KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
JX524215_KEN_2001/2           RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRSCMKCN RHNACFNVRK NADKSLILAG GSELYVTCCN NCLKK*              [185]
JX403676_KEN_2001/6           RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKSLILAG GSELYVTCCN NCLKK*              [185]
JX403675_KEN_2001/5           RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKSLILAG GSELYVTCCN NCLKK*              [185]
AY494550_TAN/2001/3           KCRAWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKK*              [185]
JX403672_KEN_2001/1           RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKSLILAG GSELYVTCCN NCLKK*              [185]
RSA_2001/2                    KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
JX403669_CAM_2002/4           KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
RSA2002/1                     KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKSTFIKQ LQPIKY* [197]
JX403666_CAM_2002/1           KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
JX403668_CAM_2002/3           KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
JX430662_GHA_2002/1           KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
JX403656_CAM_2002/5           KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
JX403663_GHA_2002/2           KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AY494553_MAL_2002/1           KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKKQMY*          [188]
JX403667_CAM_2002/2           KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AY351564_UGA_2003/1           RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKSLILAG GSELYVTCCN NCLKK*              [185]
JX403661_B_Faso_2003/1        KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
NAM_2004/3                    KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
JX403637_NAM_2004/1           KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
JX403645_RSA_2004/3           KCRTWADEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
Kenya05_Tk1                   RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYITCCN NCLKK*              [185]
NIG_01/1                      KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
Kenya06_Bus                   RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKSLILAG GSELYVTCCN NCLKK*              [185]
1546C/06                      KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112582 NIG_08/PLJs16        KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112583 NIG_08/PLJs42        KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112584 NIG_08/PLJs43        KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
JX310054_B_Faso_2007/1        KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
FJ528594_Mauritius_2007/1     TCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112585 NIG_08/BNGb2         KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112586 NIG_08/BNGb4         KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112587 NIG_08/BNGb9         KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112588 NIG_08/BNGb24        KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112589 NIG_08/BNMk42        KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112590 NIG_08/LAOk1         KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112591 NIG_08/LAOk2         KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112592 NIG_08/NW6          KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112593 NIG_08/NW10         KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
KC112594 NIG_08/NW12         KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
Vic/UGA                       RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYITCCN NCLKK*              [185]
AM712240_OURT_88_3            KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AY261360_Kenya1950            RCRTWADEKK IIILAGLNAS FEQKMFQPIV RIFPYCNWVK YIGRTCMKCN RHNACFNVRK NADKTLILAG GSELYITCCN NCLKK*              [185]
FN557520_E75_Spain            KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
AM712239_Benin_97_1_pathogenic KCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
FR682468_Georgia_2007_1       TCRTWAEEEK IIILAGLNAS FEQKMFPPIV RIFPYCSWVK YIGRTCMKCN QHNACFNVRK NADKTLILAG GSELYVTCCN NCLKNTFIKQ LQPIKY* [197]
```

NB: The isolates which appear in Table 7.1 also carry their accession numbers here in this appendix where these are available. Thus the names may be varied from the ones in the Table.