RISK FACTORS, MOLECULAR AND SEROLOGICAL EPIDEMIOLOGY OF AFRICAN SWINE FEVER IN DOMESTIC PIGS IN UGANDA

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RISK FACTORS, MOLECULAR AND SEROLOGICAL EPIDEMIOLOGY OF AFRICAN SWINE FEVER IN DOMESTIC PIGS IN UGANDA

by

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SUMMARY

African swine fever (ASF) which is caused by the African swine fever virus (ASFV) has infected domestic pigs in Uganda and several African countries. Recent events in the country where the disease is endemic show that it is progressing unabated at epidemic rates associated with a range of factors and drivers of infection.

In this study, we evaluated the drivers and risk factors, serological patterns and molecular identification of ASF in Uganda. A cross-sectional survey was conducted in seven districts of Uganda from December 2012 to April 2013, *viz.* in Pallisa, Lira, Abim, Nebbi, Kabarole, Kibaale, and Mukono which were selected to ensure wide geographic representation, had reported outbreaks in the recent past areas and were in close proximity to potentially high-risk locations associated with ASF epidemiology. A total of 196 farmers were involved in the questionnaire survey, while 190 sera samples were used for the serological assay, and 59 tissue samples were finally used for the molecular phylogenetic study.

The observed drivers, risk factors and socio-anthropological factors that were associated with ASF virus epidemiology in Uganda obtained from carefully selected representative sub-populations of pig farms and statistics in a case-control model of ASF infection on pig farms in Uganda were: the indiscriminate disposal of pig viscera and waste materials post-slaughter, farm-gate buyers collecting pigs and pig products from within the farm, retention of survivor pigs on the farm, and the disposal into an open refuse dump, of pig viscera and products following slaughter. The possession of wire mesh windows in pig houses was protective against ASF infection while the sighting of engorged ticks on the pigs, possession of a lock for each pig pen and having a gate at the farm entrance were significant.

The second component of this study cautioned against serological determination of ASF status as the sole diagnostic method with an 88 % chance of missing an on-going

infection with 6 out of 25 sera collected from Abim district testing positive while all sera from other locations were negative giving an overall prevalence of 3.1 %, and a regional prevalence of 24 %.

This work shows for the first time the use of the *TK* gene region for ASF diagnosis in Uganda. Four gene regions were successfully amplified and characterised, producing a total of 41 genomic sequences from viruses in domestic pigs in Uganda. A combination of *TK*, *p72*, *p54* and *CVR-ORF* gene regions were characterised for 10 PCR-positive domestic pigs. The *TK* gene sequencing detected four additional PCR positive individuals initially assigned a negative status on the basis of two independent *p72* assays, the OIE diagnostic PCR and C-terminal genotyping PCRs.

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Manuscripts

- Drivers and risk factors for circulating African swine fever viruses in Uganda, 2012-2013**.**

  T. Kabuuka, P.D. Kasaija, H. Mulindwa, A. Shittu, A.D.S.Bastos, F.O. Fasina

  Submitted to *Research in Veterinary Science*.

- Multi-locus typing of African swine fever viruses from domestic pigs in Uganda.

  T. Kabuuka, F.O. Fasina, P.D. Kasaija, H. Mulindwa, K.D.Atuhaire, J. van Heerden, L. Heath, A.D.S.Bastos

  In preparation

Letter

- Serological patterns of African swine fever in on-going and post-outbreak situations, Uganda

  T. Kabuuka, P.D. Kasaija, H. Mulindwa, J. van Heerden, A.D.S.Bastos, F.O. Fasina

  Submitted to *Emerging Infectious Diseases*

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LIST OF ACRONYMS

ASF	African swine fever
ASFV	African swine fever virus
CI	Confidence interval
d.f.	Degrees of freedom
DNA	Deoxyribonucleic acid
DVO	District Veterinary Office
FAO	Food and Agricultural Organisation
FAT	Fluorescent Antibody Test
FGB	Farm gate buyers
FMD	Foot and Mouth Disease
GDP	Gross Domestic Product
IB	Imunnoblotting
MAAIF	Ministry of Agriculture, Animal Industry and Fisheries
NAADS	National Agricultural and Advisory Services
NC	Negative cut-off point
NGO	Non-Governmental Organisation
OD	Optical Density
OIE	World Organisation for Animal Health
OR	Odd Ratio
P	P-value
PC	Positive cut-off point
PCR	Polymerase Chain Reaction
ROC	Receiver Operating Characteristics
STATA	General-purpose statistical software package
Std. Err.	Standard Error
UBOS	Uganda Bureau of Statistics
UN	United Nations
USD	United States Dollar
Z	Zed statistic

1 CHAPTER ONE

1.1 Literature review-Introduction

1.1.1 African swine fever virus

African swine fever (ASF), an important transboundary disease causing a devastating threat to the pig industries is caused by the African swine fever virus (ASFV) (MacLachlan *and* Dubovi, 2011). ASFV is an arthropod-borne virus belonging to the family *Asfaviridae* and genus Asfivirus and is a complex and large enveloped DNA virus with a genome of 170 – 190 kbp (Dixon *et al.,* 2005). Up to 22 different genotypes have been shown to occur in sub-Saharan Africa based on the *p72* sequences, confirming the high degree of genetic variability of the virus in its endemic setting (Boshoff *et al.,* 2007). The virus is harboured naturally in both vertebrate and invertebrate sylvatic hosts throughout sub-Saharan Africa where it is transmitted to domestic pigs when infected soft-shelled, eyeless ticks of the *Ornithodoros moubata* complex feed on them (Penrith *et al.,* 2004). The virus can survive for more than five years in competent soft tick arthropod vectors of the *Ornithodoros* genus, from which it can be transmitted to wild and domestic pigs (Oleaga-Perez *et al.,* 1990; Boinas *et al.,* 2011). While endemic African suids such as warthogs *(Phacocoerus africanus* and *P. aethiopicus),* bush pigs *(Potamochoerus larvatus* and *P. porcus)* and giant forest hogs *(Hylochoerus meinertzhangeni)* can be infected, they do not exhibit clinical symptoms (Penrith, 2009). European wild boar *(Sus scrofa)* and feral pigs as well as domestic pigs are equally susceptible to ASFV and show similar clinical signs and mortality patterns (Blome *et al.,* 2012; Penrith *et al.,* 2004).

In West Africa, the transmission cycle is never dependent on the tick vector. Studies were undertaken on a potential Ornithodoros vector in Senegal, and although a small number of the ticks tested positive for ASFV, their role if any in transmission appeared

to be minor (Vial *et al*., 2007 while in Central Africa research indicated the absence of tick involvement (Ekue & Wilkinson, 1990). In East Africa we have the sylvatic cycle as well as maintenance of the virus in the absence of wild pigs and in some areas though without the presence of ticks. Haresnape & Wilkinson (1989) showed the maintenance of ASF virus in a cycle between domestic pigs and Ornithodoros inhabiting the pigsties in Malawi.

The acute or hyperacute form of ASF in susceptible domestic pigs is characterised by a severe, haemorrhagic disease with high mortality. The incubation period is 2 - 15 days with the pigs developing a fever of 40.5 – 42°C which persists for about 4 days. This fever may be followed by inappetance, diarrhoea, incoordination and prostration. Some pigs die at this stage without other clinical signs. In other swine there is dyspnoea, vomiting, nasal and conjuctival discharge, reddening or cyanosis of the ears and snout, and haemorrhages from the nose and anus. Pregnant sows usually abort. Mortality may approach 100 % (MacLachlan & Dubovi, 2011). Subcutaneous and mucosal haemorrhages may be prominent.

The diagnosis of ASF is done through the detection of the virus, by the detection of the DNA (by PCR) or the virus antigens by FAT (direct immunofluorescence and/or antigen ELISA) and the detection of specific antibodies by indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and immunoblotting (IB) (OIE, 2012). Virus isolation (by inoculation of pig leucocyte or bone marrow cultures), is needed to confirm the virus presence, and mandatory in primary outbreaks. The control and eradication of ASFV is made difficult by several factors, including the absence of effective vaccines, marked virus resistance in environment, including its resistance in infected tissues and contaminated material and infectious animal products,

and a complex epidemiology and transmission involving ticks and wild pig reservoirs, and domestic pigs and virus interactions (Sánchez-Vizcaíno, 2012).

1.1.2 ASF outbreaks in Uganda, East Africa and international scope

Since its introduction in 1957 and 1960 in Portugal, ASF has remained endemic in the Iberian Peninsula through to the mid-1990s, and has also remained endemic in Sardinia since it was introduced in 1982. It continued spreading within Africa to countries where no outbreaks had been reported before like Madagascar and Mauritius in the Indian Ocean (Costard *et al*., 2009b). Outbreaks subsequently occurred during the 1970s in some Caribbean Islands, including Cuba and the Dominican Republic. In the 1980s the virus was reported in France, Belgium and other European countries. An outbreak was reported in Georgia in 2007 and the virus has since been found in Armenia, Azerbaijan and Russia. The disease is still enzootic in sub-Saharan Africa and Sardinia (MacLachlan & Dubovi, 2011) and been recognised as endemic in Russia for several years (Oganesyan *et al*., 2013). The virus is currently threatening other regions of the world and expanding its geographical reach at an epidemic rate (Callaway, 2012).

ASF has continuously posed devastating effects on both the commercial and subsistence pig production sectors in Africa with greater losses usually inflicted on the poorer pig producers who are less likely to implement effective prevention and control strategies or basic biosecurity measures (Edelsten & Chinombo, 1995). The disease, first reported in pigs in Kenya in 1921 (Montgomery, 1921) has been reported in Uganda, East Africa.

Uganda lies across the Equator, is landlocked and shares borders with Kenya in the east, Tanzania in the south, Rwanda in the south-west, Democratic Republic of Congo in the west (UDHS, 2006) and the newly created South Sudan in the north (Wikipedia, 2012). It has a total land area measuring 241,039 square kilometres (UDHS, 2006) with 112 administrative districts (CIA Fact Book, 2012). The climatic conditions vary due to

differences in altitude with two periods of rain (heavy in March to May and light in September to December) in the Central, Western and Eastern regions of the country. The North only experiences one period of rain per annum and therefore the agricultural potential and associated human population densities are higher in the Central and Western regions of the country (UDHS, 2006). Uganda's economy thrives mainly on agriculture (approximately 80 % of the total work force) with the majority of the population depending on subsistence farming and light agro-based industries (UBOS, 2006). In 2011, the country's GDP was estimated at USD 45.9 billion and agriculture contributed approximately 21.8 % of this total. From the 1970s to date, there has been a considerable increase in the number of semi-intensive and intensive pig units but the production system is still largely dominated by the free-range units. Specifically, between 1991 and 2008, the Ugandan pig population increased from 700,000 to approximately 3.2 million (MAAIF and UBOS, 2009; Rutebarika and Okurut, 2011). From this current figure, the Central Region has the highest population (41 %), followed by the Western and Eastern Regions (24.4 % and 22.0 %) with Karamoja sub-region, which has 1.8 % being the least (Fig 1-1).

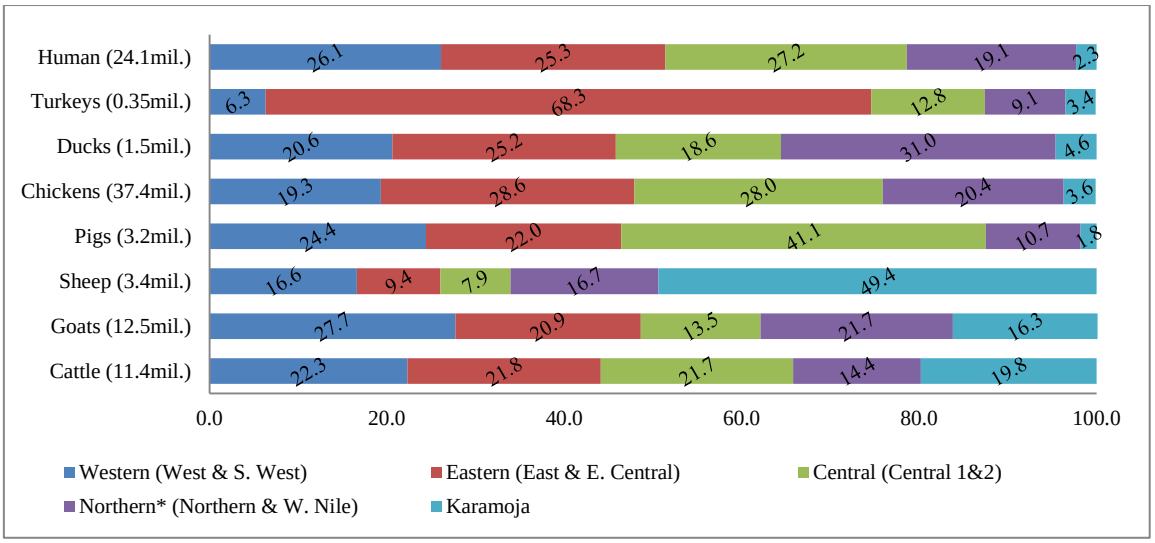

Figure 1-1: Human and animal population census (percentages/region), Uganda (UBOS, 2006; MAAIF & UBOS, 2009) **Note that all values for Northern Region are exclusive of Karamoja, a district that was excluded due to its peculiarity in terms of ruminant populations.*

African swine fever poses a major constraint to pig production in the country as evidenced by recent incessant outbreaks. At least eight major outbreaks have been reported in ten districts within the last seven years. Wakiso reported a fresh outbreak in late 2012, but more recent outbreaks occurred in Kiboga and Kabarole districts in early January 2013 (Tingira and Abigaba, personal communications). Other outbreaks have been reported previously in Adjumani and Amuru[1], Bugiri and Arua[2] in 2011, Moyo and Bundibugyo[2] and Gulu[3] in 2010, Jinja and Wakiso[4] in 2009, Masindi in 2008 and Moyo[2] in 2006. In addition, several other outbreaks have occurred within the time period (Jori *et al*., 2013).

1.1.3 Previous studies in Uganda

Scientific work on ASF in Uganda dates as far back as 1959 when the first ASF isolate from a warthog was obtained (Wesley & Tuthill, 1984). More recently in 2007, samples from domestic pigs in three districts in central Uganda were confirmed positive for ASF, using a *p72* gene-based PCR amplification assay combined with restriction enzyme analysis. Seven haemadsorbing viruses were isolated, and all were classified within the domestic pig cycle-associated *p72* and *p54* genotype IX, which also includes viruses responsible for ASF outbreaks in Kenya in 2006 and 2007, and Uganda in 2003. This availed more evidence that genetically similar ASFV within *p72* genotype IX maybe circulating between Kenya and Uganda (Gallardo *et al.*, 2011). Another study in Uganda indicated that domestic pigs, bushpigs, warthogs and soft ticks may have played various roles in the epidemiology of ASF, with some pigs being positively diagnosed with sub-clinical ASF infection (Björnheden, 2011). A previous study in West Africa also demonstrated that areas with high pig-related activities like marketing,

[1] www.thepigsite.com, 2012,
[2] www.ugandaradionetwrok.com, 2012,
[3] www.allafrica.com, 2012,
[4] www.newvision.co.ug,2012.

consumption and farming tend to have higher ASF prevalences and that a significant reduction in ASF would only be possible by fostering on-farm biosecurity protocols alongside compensation to the affected pig farmers, institution of an inclusive routine surveillance and testing system, and the reorganization of the market and transportation systems (Fasina *et al.*, 2010).

A PCR-based method that permits the detection and characterisation of ASFV field strains in 1-2 days has been applied to rapidly diagnose the disease. It involves the amplification of a 478 bp fragment corresponding to the C-terminal end of the *p72* gene. Further genetic characterisation (nucleotide sequence determination and phylogenetic analysis) may also be engaged in the definitive diagnosis of ASF (Bastos *et al.*, 2003). Specifically *p72* gene characterisation initially indicated the presence of ten major ASF genotypes on the African continent, the largest being a group of genetically homogeneous viruses recovered from outbreaks in Europe, South America, the Caribbean and West Africa (ESAC-WA genotype). However, viruses from southern and East African countries were heterogeneous with multiple genotypes found in individual countries. For instance the outbreaks of 1995 in Uganda were caused by two different viruses, UGA/1/95 and UGA/3/95 (Bastos *et al.*, 2003).

A previous assessment of field heterogeneity of isolates was done at regional level using nucleotide sequences corresponding to the C-terminal end of the *p72* gene of viruses of diverse temporal and species origin occurring in eight East African countries. The phylogenetic analysis of a homologous 404 bp region revealed the presence of thirteen East African genotypes, of which eight appeared to be country-specific (Lubisi *et al.*, 2005). An east African, pig-associated, homogeneous virus lineage incorporating strains from outbreaks in Mozambique, Zambia and Malawi over a 23-year period was shown to exist (Lubisi *et al.*, 2005). Genotype I (ESACWA) viruses were found for the first

time in the East African sylvatic hosts yet they were originally thought to exist in the West African region only where they occur in domestic pigs. The presence of discrete epidemiological cycles in East Africa and recovery of multiple genotypes showed the epidemiological complexity of ASF in this region (Lubisi *et al.*, 2005).

The central variable region (CVR) of the *9RL* open reading frame (ORF) of viruses has been characterised and used to resolve relationships between a homogeneous genotype. For instance phylogenetic analysis of 45 taxa resulted in seven discrete amino acid CVR lineages (A-G) from Malawi, Mozambique, Zambia and Zimbabwe. However, a combined *p72*-CVR analysis is required in order to first assign viruses to their genotypes and prior to intra-genotypic resolution by means of the CVR (Lubisi *et al.*, 2007). The significance of *p54* gene sequencing as an additional intermediate-resolution epidemiological tool for molecular genotyping of ASFV has also been demonstrated (Gallardo *et al.*, 2009). More recently, Atuhaire *et al.*, (2013a) elucidated the occurrence of over 300 outbreaks in a 12 year period in Uganda, further showing the eminent menace of ASF in the country. Atuhaire *et al.*, (2013b) used a combination of the *p72*, *p54* and CVR-ORF PCR analyses to perform viral discrimination for ASF outbreaks in 15 districts in Uganda from which 2 new CVR subgroups were identified.

The *TK* gene of ASF has been amplified and sequenced to show ASF virus evolutionary lineages in relation to the genotypic and pathogenic variations of the viruses. High levels of *TK* lengths and sequence heterogeneity have been displayed by East African viruses which are also known to show high *p72* diversities (Edrich 2002; Fasina *et al.*, 2013, in prep). Hernandez and Tabares (1991) showed that the *TK* gene of ASFV, which is a single-copy gene, encodes an immediate-early protein, comprising 196 amino acids and has a calculated molecular weight of 22,394. They also suggested the use of the *TK* gene for defining phylogenetic relationships among large DNA viruses.

1.2 Justification

The continuous endemic nature of ASF in Uganda warrants a concise approach if control of the disease is to be achieved at a pluralistic stakeholder level. This study therefore seeks to identify the current status using different scientific tools in the following objectives. The outcomes from the study will guide and inform preventative and control measures of ASF in Uganda

1.3 Objectives

I. To determine the drivers and risk factors for circulating ASF viruses in Uganda by focussing on the largest sector affected by ASF in Uganda, the small-scale, subsistence farmer.

II. To investigate the ASF seroprevalence in post-outbreak regions in Uganda in parallel with smaller-scale molecular prevalence estimations.

III. To genetically type the circulating strains of ASFV in Uganda using a multi-locus typing approach.

2 CHAPTER TWO

2.1 Introduction

The first investigation aimed at evaluating ASF risks in Uganda based on field surveys with the intention of identifying the risk/protective factors for disease and drivers for its transmission/prevention in the different pig production systems in Uganda. It is anticipated that the results of this study will guide decision making at policy level to support ASF control efforts in Uganda and other countries with typical piggery production systems, especially within the East African sub region.

2.2 Materials and methods

2.2.1 Study sample

A cross-sectional survey was conducted in seven districts of Uganda from December 2012 to April 2013. These locations were: Pallisa, Lira, Abim, Nebbi, Kabarole, Kibaale, and Mukono (Fig 2-1). These study areas were purposely selected to ensure wide geographic representation of all regions in Uganda namely the East, North, Karamoja, West Nile, West, South West and the Central regions. Secondly, for districts that had reported outbreaks in recent past (an outbreak in this regard refers to a situation where unusual mortalities had been observed in a pig population and was investigated by veterinary officers, serum and tissue samples were collected, tested and confirmed in the laboratory as serologically positive for ASF antibodies or positive viral genome presence by means of a *p72* gene diagnostic PCR). Finally, these districts were carefully selected to represent areas in close proximity to potentially high-risk locations associated with ASF epidemiology, such as game parks, major pig consumption areas and trade or marketing routes, and forest reserves. Farms where such positive genetic materials were detected with or without positive serology were declared as case farms and any farms with a similar or deviating clinical case report and history but whose

samples were consistently negative for ASF genetic materials and serology were declared as control farms. The unit of interest for this study was an individual pig farm where an outbreak had occurred previously or within the vicinity.

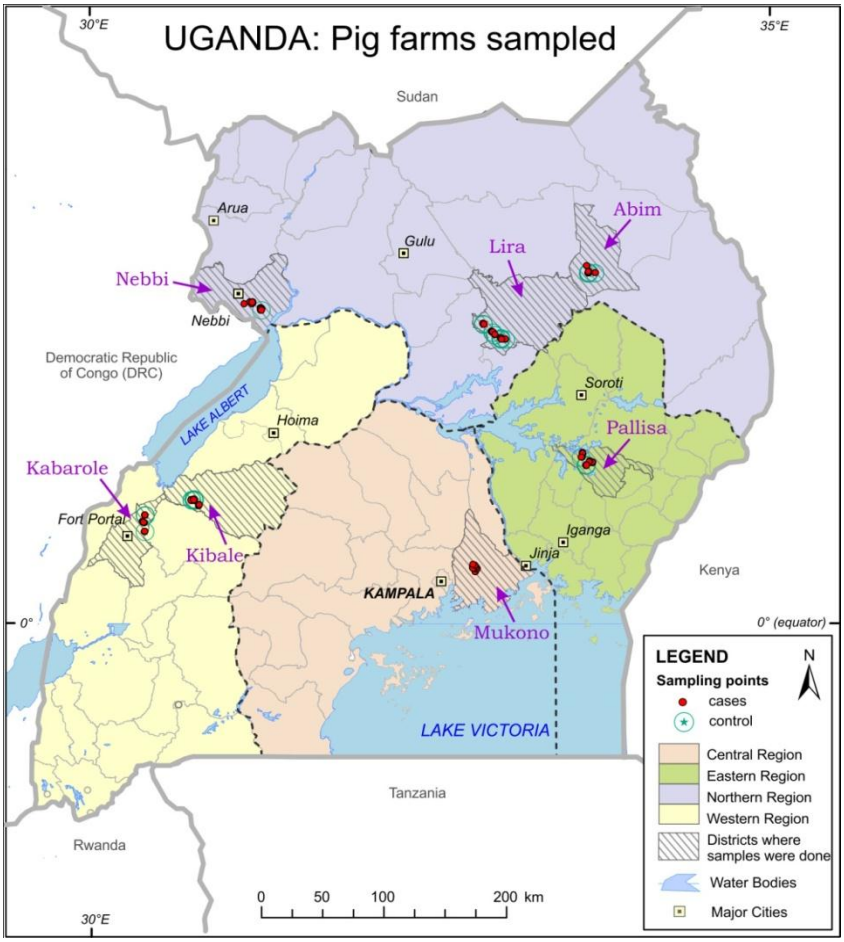

Figure 2-1: Map of Uganda showing study sites (cases and control farms per region), 2012-2013

2.2.2 Sample size determination

Making an assumption that sampling would be from a large population and that a simple random sampling design would be followed, Epi Info® 6 was used to calculate the sample size based on the exact binomial distribution (Fosgate, 2009). It was estimated that 193 pig farms would need to be sampled for an estimated 50 % prevalence at 95 % confidence with 10 % precision and a design effect of 2 to account for clustering within districts. For equal representation amongst the sampled

populations, 28 respondents from each of the previously defined farms were selected per district to be interviewed for the questionnaire survey. Within the districts, sub counties served as the primary sampling units, and villages represented the secondary sampling units. These were selected randomly using a multi-stage sampling approach.

2.2.3 Data collection

A questionnaire on the pig farmer, farm demographics, risk factors and self-reported farm-level biosecurity variables was developed and evaluated at the Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria. It was pre-tested with five farmers by two interviewers in Tororo, Uganda and adjusted to fit the survey purpose. Three interviewers were ultimately recruited to administer the questionnaire within each district after a self-explanatory letter of consent was submitted to each respondent and signed to confirm their willingness to avail their personal and farm information (See Appendix). Farmers who had reported outbreaks and those whose farms were confirmed ASF-positive were asked additional questions regarding post-outbreak management and behaviour.

2.2.4 Data entry and analysis

Data coding, entry and filtering were done using EpiData® 3.1 and data were exported into STATA® 9 for analysis (Stata Corporation, Texas, USA). A combination of Open Epi® Version 2.3 and STATA® 9 was used to carry out the univariable regression analyses, multivariable logistic regression analysis and descriptive statistics.

2.3 Results and analysis

2.3.1 Descriptive statistics

2.3.1.1 Pig farmer demographics

A total of 196 farmers were involved in the survey. Four variables were used to describe farmer demographics including age of the respondent, level of education, main occupation and approximate mean time dedicated to pig farming per day. The majority of farmers interviewed were within the age range of 31-40 years (n=97; 49.49 %) while 27.55 % fell within the age category of 21-30 years (n=54) and only 4.08 % were under 20 years of age (n=8) with none in the greater than 50 years category. Similarly 48.98 % of the farmers had secondary-level education (n=96) or primary level education (n=89; 45.41 %), while nine had post-secondary schooling (4.59 %) and only two respondents (1.02 %) had university education. Ninety percent (90.31 %) of the farmers regarded piggery as their principal occupation (n=177) while the remainder also practised crop husbandry (n=9; 4.59 %) or other farming/business activities (n=10; 5.10 %) in addition to piggery. Over half of the total respondents dedicated 1-2 hours daily to pig farming (n=102; 52.04 %), 60 spent between 3-4 hours (30.61 %) and 34 spent less than 1 hour (17.35 %) (Fig. 2-2).

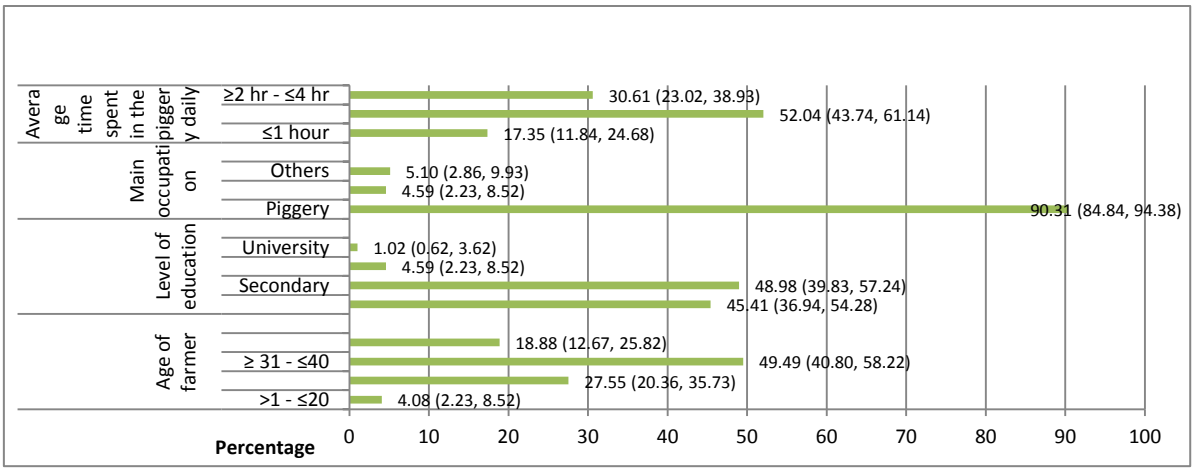

Figure 2-2: Pig farmers' demographics (in percentages) for selected pig farms in Uganda, 2012 to 2013 (*Confidence intervals of true values were taken at 95 % using Mid-P Exact method.)*

2.3.1.2 Farm demographics

Three variables were used to describe farm demographics namely the breed of pigs, average herd size and source of pigs (Fig. 2-3). Approximately half of the farmers kept mixed breeds of pigs (n=97; 49.49 %) while others kept the local breed (n=53; 27.04 %) and only 23.47 % kept exotic breeds (n=46). Approximately 89.80 % of farmers had an average herd size of 1-10 pigs (n=176) and the remainder had 11-50 pigs on average (n=20; 10.20 %). Sixty eight per cent of the farmers obtained new stock of pigs from neighbouring farms (n=134) while others sourced replacement pigs from the markets (n=53; 27.04 %) and Government or non-governmental organisations (NGOs) projects (n=9; 4.59 %). Farmers did not breed their own replacement stock.

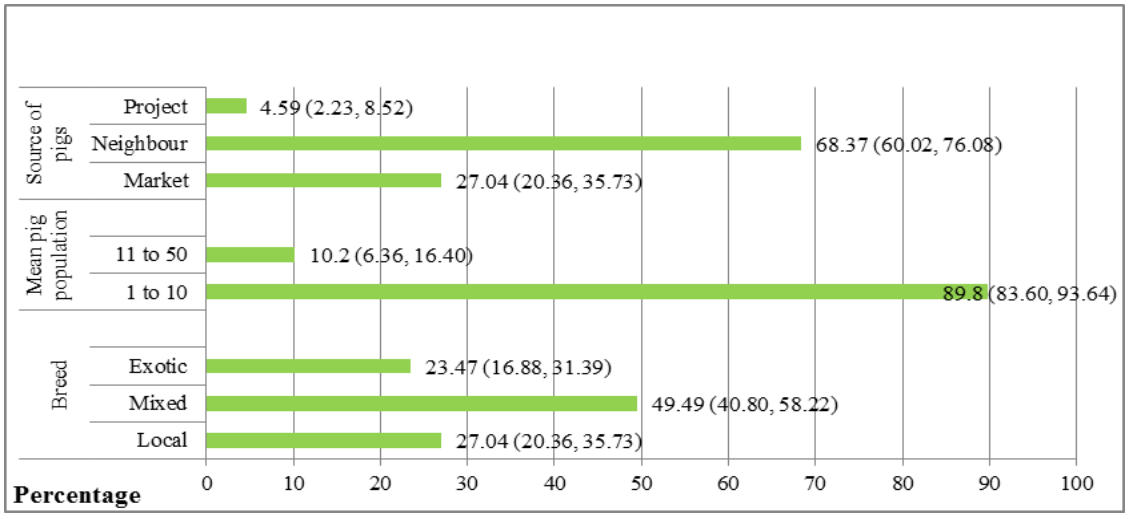

Figure 2-3: Pig farm demographics (in percentages) for selected pig farms in Uganda, 2012-2013 (*Confidence intervals of true values were taken at 95 % using Mid-P Exact method.)*

2.3.2 Univariable logistic regression analysis

2.3.2.1 Risk factors for infection of farms with ASF virus

A total of sixteen variables were analysed in the univariable logistic regression for risk factors of ASFV infection in farms (Table 2:1). The following variables were significant at $P \leq 0.25$ and were considered for inclusion in the final multivariable logistic regression model: Farm-gate buyers visited farms to collect products, pig farmers

provided source of water to pigs, farmers kept survivor pigs on the farm, farmers sighted engorged ticks on pigs, and farmers disposed of pig viscera by burning, dumping in refuse pit or indiscriminately.

Table 2-1.Univariable logistic regression analysis of risk factors associated with ASF outbreaks in pig farms, Uganda 2012 -2013

Variable	Category	Case (%)	Control (%)	OR	95 % CI	*P*-value
Farm-gate buyers collected pig products from farm	No	18(41.86)	25(58.14)	1.00	*Reference*	*NA*
	Yes	122(79.74)	31(20.26)	5.41	2.63, 11.32	<0.001
Pig farmer visited other farms	No	16(11.43)	4(7.14)	1.00	*Reference*	*NA*
	Yes	124(88.57)	52(92.86)	0.60	0.17, 1.79	0.39
Pig farmers provided source of water	No	4(2.86)	4(7.14)	1.00	*Reference*	*NA*
	Yes	136(97.14)	52(92.86)	2.60	0.57, 11.92	0.21
Pig farmers shared same water source	No	122(87.14)	50(89.29)	1.00	*Reference*	*NA*
	Yes	18(12.86)	6(10.71)	1.23	0.47, 3.56	0.71
Farmer kept any survivor pigs	No	112(80.00)	54(96.43)	1.00	*Reference*	*NA*
	Yes	28(20.00)	2(3.57)	6.71	1.78, 43.15	0.002
Farmer sighted engorged ticks on pigs	No	136(97.14)	52(92.86)	1.00	*Reference*	*NA*
	Yes	4(2.86)	4(7.14)	0.38	0.08, 1.76	0.21
Farmer's pig products disposal/sale method	Market	86(61.4)	34(60.7)	*1.00*	*Reference*	*NA*
	Farm Buyers	30(21.4)	14(25)	0.85	0.38,1.95	0.664
	Slaughter	24(17.2)	8(14.3)	1.19	0.45,3.2	0.708
Source of feeds	Own	95(67.9)	39(69.6)	*1.00*	*Reference*	*NA*
	Buy	29(20.7)	9(16.1)	1.3	0.54,3.8	0.511
	Pig roams	16(11.4)	8(14.3)	0.8	0.3,2.41	0.676
Disposal method of pig viscera	Sell for consumption	4(2.9)	7(12.5)	1.00	*Reference*	*NA*
	Burn	24(17.1)	14(25)	3	0.62,16.2	0.114
	Dump in refuse pit	60(42.9)	25(44.6)	4.2	0.95,21	0.024
	Indiscriminate	52(37.1)	10(17.9)	9.1	1.83,48.9	0.0006

2.3.2.2 Self- reported on-farm biosecurity

A total of 27 variables were used for the univariable regression of self-reported on-farm biosecurity parameters (Table 2:2). The following variables were significant at $P \leq 0.25$ and were considered for inclusion in the final multivariable logistic regression model: gate was present at farm entrance, wire mesh window was used on pig housing structure, some farm records were kept, sufficient feeding and watering spaces were available for all pigs, usage of disinfectant after cleaning is done routinely, presence of lock for each pig pen, farmer assessed health status of pigs coming into the farm and consulted with a veterinarian in case of sick pigs.

Table 2-2.Univariable logistic regression analysis of self-reported biosecurity associated with ASF outbreaks in pig farms, Uganda 2012 -2013

Variable	Category	Case (%)	Control (%)	OR	95 % CI	P-value
Restricted access to all visitors	No	90(64.29)	36(64.29)	1.00	*Reference*	*NA*
	Yes	50(35.71)	20(35.71)	1	0.52, 1.93	0.99
Fenced premises	No	46(32.86)	15(26.79)	1.00	*Reference*	*NA*
	Yes	94(67.14)	41(73.21)	0.75	0.39, 1.48	0.42
Gate at entrance	No	87(62.24)	40(71.43)	1.00	*Reference*	*NA*
	Yes	53(37.86)	16(28.57)	1.52	0.78, 3.04	0.22
Wire mesh window	No	119(85.00)	40(71.43)	1.00	*Reference*	*NA*
	Yes	21(15.00)	16(28.57)	0.44	0.21, 0.94	0.04
Record keeping	No	22(15.71)	15(26.79)	1.00	*Reference*	*NA*
	Yes	118(84.29)	41(73.21)	1.96	0.91, 4.14	0.08
Food and water control	No	16(11.43)	8(14.29)	1.00	*Reference*	*NA*
	Yes	124(88.57)	48(85.71)	1.29	0.49, 3.19	0.58
Terminal (end of operation) cleaning	No	63(45.00)	30(53.57)	1.00	*Reference*	*NA*
	Yes	77(55.00)	26(46.43)	1.41	0.75, 2.64	0.28
Routine (regular) cleaning	No	42(30.00)	17(30.36)	1.00	*Reference*	*NA*
	Yes	98(70.00)	39(69.64)	1.02	0.51, 1.99	0.95
Safe disposal of faeces and dead pigs	No	44(31.43)	14(25.00)	1.00	*Reference*	*NA*
	Yes	96(68.57)	42(75.00)	0.73	0.35, 1.46	0.38
Quarantine newly purchased pigs for at least 10 days	No	62(44.29)	29(51.79)	1.00	*Reference*	*NA*
	Yes	78(55.71)	27(48.21)	1.35	0.72, 2.53	0.35
Regular cleaning and disinfection of feeders and drinkers	No	42(30.00)	17(30.36)	1.00	*Reference*	*NA*
	Yes	98(70.00)	39(69.64)	1.02	0.51, 1.99	0.95
Sufficient feeding and watering space available for all pigs	No	35(25.00)	19(33.93)	1.00	*Reference*	*NA*
	Yes	105(75.00)	37(66.07)	1.54	0.78, 3.01	0.22
Sufficient space for each pig (No overcrowding)	No	38(27.14)	18(32.14)	1.00	*Reference*	*NA*
	Yes	102(72.86)	38(67.86)	1.27	0.64, 2.49	0.49
Removed manure and litter routinely	No	40(29.67)	17(30.36)	1.00	*Reference*	*NA*
	Yes	100(71.43)	39(69.64)	1.09	0.54, 2.14	0.80
Used disinfectant after cleaning	No	123(87.86)	54(96.43)	1.00	*Reference*	*NA*
	Yes	17(12.14)	2(3.57)	3.71	0.94, 24.5	0.06
Lock for each pen	No	73(52.14)	35(62.50)	1.00	*Reference*	*NA*
	Yes	67(47.86)	21(37.50)	1.53	0.81, 2.92	0.19
Assessed health status of pigs coming in farm	No	23(16.43)	15(26.79)	1.00	*Reference*	*NA*
	Yes	117(83.57)	41(73.21)	1.86	0.87, 3.90	0.11
Never mixed different ages	No	56(40.00)	19(33.93)	1.00	*Reference*	*NA*
	Yes	84(60.00)	37(66.07)	0.77	0.40, 1.47	0.44
Never mixed different species	No	80(57.14)	27(48.21)	1.00	*Reference*	*NA*
	Yes	60(42.86)	29(51.79)	0.70	0.37, 1.31	0.26
All-in all-out production	No	128(91.43)	50(89.29)	1.00	*Reference*	*NA*
	Yes	12(8.57)	6(10.71)	0.78	0.28, 2.37	0.64
Consulted with a veterinarian in case of sick pigs	No	2(1.43)	5(8.93)	1.00	*Reference*	*NA*
	Yes	138(98.57)	51(91.07)	6.69	1.28, 51.11	0.02
Changed rubber boots/slippers	No	124(88.57)	47(83.93)	1.00	*Reference*	*NA*
	Yes	16(11.43)	9(16.07)	0.68	0.28, 1.70	0.39
Washed /disinfected equipment and tools	No	78(55.71)	31(55.36)	1.00	*Reference*	*NA*
	Yes	62(44.29)	25(44.64)	0.99	0.53, 1.85	0.96
Pest control (rodents and insects)	No	51(36.43)	19(33.93)	1.00	*Reference*	*NA*
	Yes	89(63.57)	37(66.07)	0.90	0.461, 1.72	0.75
Prompt sick/dead bird disposal from the farm	No	11(7.86)	7(12.50)	1.00	*Reference*	*NA*
	Yes	129(92.14)	49(87.50)	1.67	0.58, 4.58	0.33
Changed solutions in foot pans regularly	No	135(96.43)	53(94.64)	1.00	*Reference*	*NA*
	Yes	5(3.57)	3(5.36)	0.66	0.15, 3.44	0.58
Audited	No	64(45.71)	25(44.64)	1.00	*Reference*	*NA*
	Yes	76(54.29)	31(55.36)	0.96	0.51, 1.79	0.90

2.3.3 Multivariable logistic regression analysis

An explanatory model for multivariable logistic regression analysis was designed for this study and all 15 variables that were considered significant in the univariable logistic regression analysis at $P \leq 0.25$ were considered. A backward elimination procedure was used to exclude the factors one at a time based on non-plausibility. Only 8 variables were retained in the final multivariable logistic regression model at $P \leq 0.05$ (Table 2:3).

Table 2-3.Multivariable logistic regression of variables associated with ASF outbreaks in pig farms, Uganda, 2012 – 2013

Variable	OR	Std. Err.	z	P	95 % CI
Indiscriminate disposal of pig intestines and waste materials after slaughter procedure	71.9	63.2	4.87	0.000	12.9,402
Farm-gate buyers collecting pig and pig products within farm	23.8	13.9	5.40	0.000	7.53,74.9
Survivor pig kept by farmer	18.6	23.3	2.33	0.020	1.59,17.2
Gate at entry of farm	14.1	11.8	3.18	0.001	2.76,72.2
Refuse dump disposal of pig intestines and waste materials after abattoir procedure	9.5	6.17	3.49	0.000	2.69,33.9
Lock for each pig pen	9.5	6.79	3.15	0.002	2.34,38.5
Wire mesh window on housing structure	0.1	0.05	-3.23	0.001	0.01,0.30
Engorged ticks seen on pig	0.01	0.02	-3.32	0.001	0.00,0.16

The most plausible risk factors with association to ASFV infection of pig farms in Uganda were indiscriminate disposal of pig viscera and waste materials after slaughter (OR = 71.9; $CI_{95\ \%}$ = 12.9, 402; P<0.001) and farm-gate buyers collecting pig and pig products within the farm (OR = 23.8; $CI_{95\ \%}$ = 7.53, 74.9; P<0.001). Other risk factors of significance included retention of survivor pigs on the farm (OR = 18.6; $CI_{95\ \%}$ = 1.59, 17.22; P=0.020), having a gate at the entrance of the farm (OR = 14.11; $CI_{95\ \%}$ = 2.76, 72.2; P=0.001), disposal of pig viscera and products following slaughter into an open refuse dump (OR = 9.5; $CI_{95\ \%}$ = 2.69, 33.9; P<0.001), possession of a lock for

each pig pen (OR = 9.5; CI = 2.34, 38.5 P=0.002); The possession of wire mesh window in pig house (OR = 0.1; $CI_{95\ \%}$ = 0.01, 0.30; P=0.001) and sighting of engorged ticks on the pig (OR = 0.01; $CI_{95\ \%}$ = 0.0, 0.16; P=0.001) were protective factors for ASF in this study. The Hosmer-Lemeshow goodness-of-fit X^2 was 1.91 (d.f. = 6), P = 0.928). Two variables, namely consultation with a veterinarian when animals are sick and provision of source of water were collinear (Φ = -0.794; P $\geq$ 0.8). The latter was eliminated in the multivariable logistic regression model due to collinearity and the former due to non-significance. A curve was plotted to show the distribution of variables post estimation (Fig. 2-4).

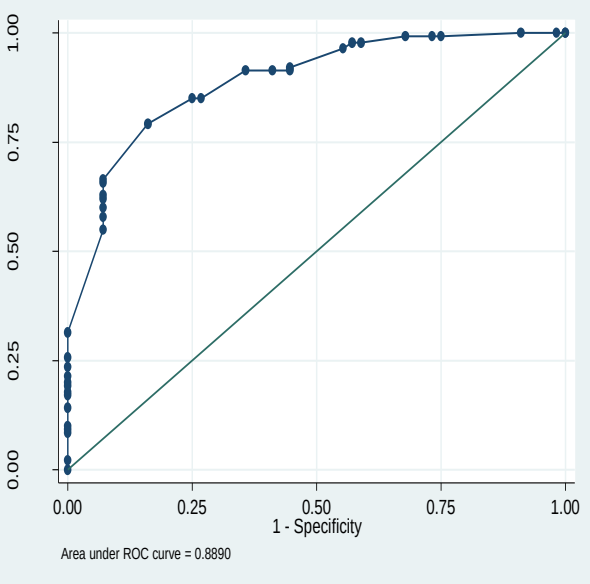

Figure 2-4: Receiver Operating Characteristics (ROC) post estimation curve after logistic regression

2.3.4 Outbreak farms: Perception and post outbreak behaviour of farmers

A total of 140 farmers reported prior outbreaks on their farms and 8 variables were used to describe parameters on these farms (Table 2:4). Seventy nine percent of the farmers reported having had an outbreak on their farm in 2012 (n=110) while 15 % had an outbreak in 2011 (n=21) and 6 % in 2010 (n=9). Nearly half of the farmers notified the District Veterinary Officer (DVO) when they realized the outbreak (n=68; 48 %), while

43 % reported to the local Veterinarian (n=60), with only 6 % reporting to animal husbandry officers (n=8) and the remaining 3 % not notifying authorities (n=4). The period between observation of clinical signs of disease and reporting it to the authorities was approximately 1-2 weeks for 79 farmers (56 %); while it took 1-7 days for 56 farmers (40 %) and one month for 4 farmers (3 %). Only one farmer (<1 %) reported within 24 hours of noticing the clinical signs. With regard to the ease of reporting clinical signs of ASF by the farmers, 75 % indicated that it was not easy (n=105), 21 % confirmed that it was very difficult (n=30) and approximately 4 % stated that it was easy to make the necessary contact for reporting (n=5).

Half of the farmers perceived that roaming pigs were responsible for the introduction of infection onto their farms (n=70; 50 %), while 24 % attributed the cause to other farmers visiting their premises (n=33). Others indicated their own pigs roamed prior to infection (n=24; 17 %), variable causes (n=10; 7 %) or wild pigs (n=3; 2 %). Sixty eight percent of farmers were not sure of the status of infection of the neighbouring pig farms (n=95), five percent (n=7) were sure that there was no infection in the neighbourhood while 27 % (n=38) were sure of infected farms within the neighbourhood.

On the significance of ASF for infected farms, 64 % of farmers reported that ASF has affected their farms mainly through the loss of pigs (n=89), while 18 % suffered reduced income (n=25), totally lost income (n=22; 16 %) or incurred more costs for disease prevention (n=4; 2 %). Post-outbreak of ASF, 94 % of the farmers re-stocked their farms (n=132), 4 % tried other livestock (n=5), 1 % abandoned piggery (n=2) and less than 1 % tried another means of making a living (n=1).

Table 2-4.Cross tabulation of perception and post-outbreak behaviour of farmers in ASF outbreak locations, Uganda, 2012-2013

Farmers reporting outbreaks n = *140*, Frequency (%)					
	Personnel that was notified				
Year of reported outbreak	Local Veterinarian	DVO	No one	Other	Total (%)
2010	6(66.67)	3(33.33)	0(0.00)	0(0.00)	**9(6)**
2011	8(38.10)	12(57.14)	1(4.76)	0(0.00)	**21(15)**
2012	46(41.82)	53(48.18)	3(2.73)	8(7.27)	**110(79)**
Total	**60(43)**	**68(48)**	**4(3)**	**8(6)**	140
	Ease of report of ASF by farmer				
How long it takes farmer to detect disease and report to authorities		Easy	Not easy	Very difficult	Total
24 hr		0(0.00)	1(100.00)	0(0.00)	**1(1)**
1-7 days		2(3.57)	50(89.29)	4(7.14)	**56(40)**
1-2 weeks		3(3.80)	51(64.56)	25(31.65)	**79(56)**
1 month		0(0.00)	3(75.00)	1(25.00)	**4(3)**
Total		**5(4)**	**105(75)**	**30(21)**	140
	Infected farms in neighbourhood				
What farmer thinks is responsible for infection on their farm		Yes	No	Not sure	Total
Visiting farmers		6(18.18)	1(3.03)	26(78.79)	**33(24)**
Wild pigs		1(33.33)	0(0.00)	2(66.67)	**3(2)**
Roaming pigs		15(21.43)	6(8.57)	49(70.00)	**70(50)**
Own pig roaming		9(37.50)	0(0.00)	15(62.50)	**24(17)**
Other		7(70.00)	0(0.00)	3(30.00)	**10(7)**
Total		**38(27)**	**7(5)**	**95(68)**	140
	Farmer's reaction after ASF outbreak				
Effect ASF had on farm	Abandoned piggery	Re-stocked	Tried other stock animals	Other	Total
Lose pigs	2(2.25)	84(94.38)	2(2.25)	1(1.12)	**89(64)**
Lose income	0(0.00)	21(95.45)	1(4.55)	0(0.00)	**22(16)**
Reduction in income	0(0.00)	23(92.00)	2(8.00)	0(0.00)	**25(18)**
More costs for disease prevention	0(0.00)	4(100.00)	0(0.00)	0(0.00)	**4(2)**
Total	**2(1)**	**132(94)**	**5(4)**	**1(1)**	140

2.4 Discussion

In this study, the drivers and risk factors of ASF infection on pig farms in Uganda were assessed using three comprehensive parameters including the general farm demographics, risk and protective factors as well as post-outbreak perceptions and behaviours of farmers. While the first category describes the characteristics of pig farmers and the production management systems, the others identify the pre-eminent factors associated with or protective of ASF outbreaks on farms, and farmers' perceptions including reactions that serve as drivers of ASF infection of new farms.

2.4.1 Drivers and risk factors of ASF in Uganda

2.4.1.1 Descriptive study

The large majority of pig farmers were within the age range 21-40 (77 %) and had a maximum of either primary or secondary school education (94 %). Similarly, a large percentage (90 %) operated a piggery as their main occupation. Since these sectors of the population are not completely illiterate but at the same time will not be able to effectively utilise highly technical documents, programmes and policies on animal health, such documents and biosecurity extension services should be clear, simple and unambiguous, and targeted to a relatively youthful population. The use of learning aids, pictorial guides and other participatory epidemiology tools should be encouraged when conferring basics of piggery management and biosafety information to farmers in Uganda. It is interesting to know that small-scale pig farmers were interested in upgrading to larger scale operations with improved management and biosecurity principles. Certain farmers had indeed acquired loans to improve pig housing facilities from simple wooden pens to those with concrete floors and wire mesh windows. Similarly, some farms especially in Kabarole district built their sties on platforms approximately 1.5 m off the ground to improve hygiene (Fig 2-5). This evidence of

improved hygiene and management practices can be positively explored and enhanced with sustained training to reduce mortality associated losses from pig diseases and improve sources of income to farmers in resource-poor settings.

There was a preferential tendency for improved breeds of pigs and mixed breeds were kept by approximately 50 % of the interviewed farmers. It is the principal opinion of farmers that mixed breeds offered better production, reproduction and mothering traits in comparison to the primarily local or exclusively exotic pigs in the Ugandan setting. In addition, exotic breeds of pigs were viewed as expensive and scarce.

Figure 2-5: Pig pen built off the ground.

Figure 2-6: Survivor pig kept in isolation" evidently near piglets on the left.

Figure 2-7: Typical wood pig housing structure, with door at entrance only, and no fence.

Pigs have previously been identified as a means of income generation, food security and social security among the rural and peri-urban poor (Dietze, 2011). In this study, approximately 90 % of the famers had an average herd size of less than ten animals, an indication that this venture was a subsistence, "instant money bank" form of financial security or a bandwagon effect of neighbourhood farmers' situations. Though no specific question was asked with regard to the living standards of the participating farmers, it was observed that the majority of these farmers were poor and would need more inputs from government and NGOs to become semi-intensive or commercial. In Uganda, both the government and other development partners have previously assisted farmers with start-up materials (stock, funds, facility, and information) but in this study, we realised that only about 5 % of interviewed farmers had benefited from such programmes, an indication of low uptake. It may be necessary to revise the existing programmes to reach or accommodate more small-scale and emerging farmers. The majority of farmers got their pigs from neighbouring farms (68 %), whose disease statuses were unknown, or from markets (27 %) which are usually collection areas and have been identified as sources of disease re-distribution and dissemination (Costard *et al.*, 2009; Fasina *et al.*, 2010). It should be noted that many of the interviewed farmers previously confirmed having kept back ASF survivor pigs (Fig 2-6) or to have sold same and sick pigs to the live animal markets. The contributions of such actions to the epidemiology of ASF in Uganda and neighbouring countries cannot be overemphasised and will be discussed in greater detail as an important risk factor for ASFV infection.

2.4.1.2 Risk and protective factors

Indiscriminate disposal of pig intestines and waste materials post-slaughter is the most significant risk factor that is associated with or influencing ASF infections of farms (OR = 71.9; $CI_{95\ \%}$ = 12.9, 402; P<0.0001). Home slaughtering of sick and untested animals

together with indiscriminate disposal of viscera may disseminate ASFV to clusters of neighbouring farms especially through fomites and scavengers. In Uganda, the lack of well-established abattoir systems that would ensure safe disposal of pig wastes after slaughter as well as the lack of awareness on the mode of transmission of ASFV is likely a strong driver of disease dissemination. Fasina *et al.,* (2012) have recently established links between these factors and ASF incidence. The mode of pig viscera disposal is crucial for the control of the disease since different strategies need to be crafted for farmers who dispose indiscriminately compared to those who collect and dispose of in on refuse dump. The knowledge of basic animal husbandry (management and health practices) may be insufficient amongst the largely literate population (94 % had up to secondary school education). Increased sensitisation of these literate pig farmers by the local and regional animal health authorities and regular veterinary extension services will be important in this regard.

The collection of pigs and pig products directly from the farm (Fig 2-7) by farm-gate buyers (FGB) was also significantly associated with ASF infection of farms (OR = 23.8; $CI_{95\ \%}$ = 7.53, 74.9; P<0.001) highlighting a causal relationship existing between infected farms and movements within the farms. Sixty eight per cent (68 %) of the respondents cannot confirm if the neighbours had infections on their farms and an additional 27 % confirmed such neighbourhood infections. The FGB enter farms to collect pigs, some of which may be infected, and subsequent visits to naïve pig populations within the neighbourhood may seed infection inadvertently. Such dissemination may be carried from farm to farm or along the market routes. In addition, in the case of an outbreak, movement in and out of the farms is often uncontrolled and infected farm owners may sometimes lead the FGB to other largely non-gated farms in the neighbourhood.

In our analysis, the presence of a gate at point of entry of farm (OR = 14.11; $CI_{95\ \%}$ = 2.76, 72.2; P=0.001) (Fig 2-7) and the presence of a lock for each pig pen (OR = 9.5; $CI_{95\ \%}$ = 2.34, 38.5 P=0.002) were significantly associated with ASF infection and outbreaks on the farm. Although Vaillancourt and Carver, (1998) and Racicot *et al.*, (2011) had previously established that the presence of a gate and other biosecurity measures do not always correlate with use and compliance, response bias with regard to biosecurity questions in farms may have played a role in this particular response (Nespeca *et al.*, 1997). In addition, it is instructive to know that certain farms had open fences and gates which could not prevent scavenging and roaming pigs from entering onto farms. Other workers similarly established the neighbourhood effect and the role of FGB in the spread of pig diseases (Fritzemeier *et al.*, 2000; Anon, 2011; Penrith *et al.*, 2012).

Furthermore, the presence of survivor pigs (Fig 2-6) usually kept back with other pigs was significantly associated with ASF infection (OR = 18.6; $CI_{95\ \%}$ = 1.59, 17.22; P=0.020) and about 15 % of the surveyed farmers indicated that they had kept survivor pigs. Arias and Sánchez-Vizcaíno (2002), observed that a less virulent strain of ASF can lead to apparently healthy carriers that subsequently play important roles in the endemicity and dissemination of ASF. It must be noted that some viruses of lower pathogenicity were vaccine viruses that were very widely disseminated in the Iberian Peninsula and should not be confused with those in areas where such vaccine viruses have never been distributed. Certain districts had large populations of survivor pigs and there were unconfirmed claims that the local breed withstood disease adversities better and had a higher percentage of survivors. Though no scientific evidence has been ascribed to this observation, it is possible that an intrinsic environmental-associated adaptability is making the local pigs respond better to ASF infection especially in naïve

farms or that less-virulent viruses are co-circulating with highly virulent forms in the field. It is more than possible that the inherent resistance seen in pigs is at least partly due to being exposed to less virulent viruses, with pigs infecting others with less virulent or avirulent ASF virus and actually vaccinating them, without starting outbreaks. As an integral part of this study (See Chapter 3), the full genotyping of all isolates from the current outbreaks was done and results may shed light on this observation.

The refuse dumps also serve as drivers for infection since the disposal of pig viscera and waste materials post-slaughter into refuse dumps was found to have significant association with ASF infection (OR = 9.5; $CI_{95\ \%}$ = 2.69, 33.9; P<0.001). Refuse dumps are openly exposed in many areas of Africa and this creates possibilities for pathogen transfer from them. Scavenging animals especially pigs visit these dumps and may carry infections back to the farms, or contaminate surfaces, food and water. Though costly, the use of effective disinfectants and deep burying of carcasses and wastes from suspect animals should be done as a routine. Slaughterhouses should also be encouraged to have underground sewer systems for waste disposal (Sánchez-Vizcaíno *et al.*, 2012).

The presence of wire meshed-windows on improved pig houses was protective against ASF infection (OR = 0.1; $CI_{95\ \%}$ = 0.01, 0.30; P=0.001). The wire mesh window possibly reduces contact between free flying birds, rodents or other animals and domestic pigs; and also limits human contact. This protective tool should therefore be encouraged among small scale pig farmers. The mesh should be of adequate size to keep out the stable fly, *Stomoxys calcitrans* that has been shown to have ability for virus transmission for up to 48 hours (Mellor *et al.*, 1987). Similarly, the presence of engorged hard ticks on pigs showed an association to ASF infection (OR = 0.01; $CI_{95\ \%}$ = 0.0, 0.16; P=0.001) that seemed protective though the immediate reason for this

association cannot be explained. These hard ticks were collected where available and were processed using molecular virology approaches. Whilst the presence of ticks on pig bodies is generally considered an indicator of poor management it is likely to be widespread since these farmers hardly practiced parasite control, thus their detection may be an indirect indicator of which small-scale farmers are fully engaged in pig-keeping and are therefore keenly observant of the status of their pigs. This possibility explains what would otherwise be a puzzling negative association with ASF infection as it is this segment of farmers that is also likely to take additional precautions to ensure pig health.

Overall, almost all of the self-reported biosecurity measures employed by farmers were broadly ineffective in this assessment. We are aware that ASF eradication without vaccination is difficult but possible; this will depend largely on the commitment from government to compensate affected farmers, the effectiveness of reporting, good networking of veterinary infrastructures, as well as strict adherence to biosecurity by farmers and other role players (Sánchez-Vizcaíno *et al.*, 2012).

2.4.1.3 Post-outbreak perceptions and reactions

In this study, more outbreaks were reported in interviews for the year 2012 compared to the previous two years. Though this observation can be attributed to recall/memory bias since record keeping skills of farmers were poor, it may also be due to increasing awareness of the disease and the need for more reporting or an increasing presence of the disease on farms. Some farmers posited that although they have seen clinical signs indicative of ASF, they never reported this to the authorities and in the course of our study, we observed that one sub-county of a district previously considered as not infected had occurrence of dying pigs without the knowledge of the local veterinarian at the time. It is also important to realise that the dynamics of ASF in Uganda are changing

rapidly as Nebbi, Kabarole, Abim and Lira, all previously considered to be non-infected locations (Boqvist and Stahl, 2010) all have positive farms and some were under quarantine at the time of the study.

Though the Veterinary Sections of the Districts engage pig farmers and have records of reported outbreaks at the various District headquarters, these may not be truly representative of the field situation. Our evaluation and the report from the previous study by Boqvist and Stahl (2010) confirmed these disparities. In addition, such inconsistencies in the exact numbers of outbreaks and confirmed data have been known to occur due to poor disease awareness (Costard *et al.*, 2009). The majority of the farmers (91 %) notified both the DVO and local veterinarians on recognition of abnormal signs in pigs. Since the few designated veterinarians cannot realistically cover all of the districts and administrative areas effectively, the role of veterinary paraprofessionals in the rapid syndromic surveillance and diagnosis becomes extremely important. This being the case, these individuals should be trained in the acts of disease recognition, rapid diagnosis, outbreak control, management and associated biosecurity under the supervision of competent veterinarians. While approximately 75 % of the surveyed farmers stated that they had some difficulty reporting syndromes observed on their farms, another 21 % claimed that such reporting was extremely difficult due to ignorance of the disease and how it presented.

A total of 139 farmers (99 %), took between 7 days and up to a full month to report signs of unusual signs and death in the pig populations to the designated authorities. In instances of an outbreak, the officials inform the DVO who institutes an investigative team that usually recommends quarantine where positive animals are identified. Delayed reporting has an implication with regard to infection of new locations. Necessary logistic support to enhance rapid reporting and the use of available structures

including the National Agricultural Advisory Services (NAADS) and other extension will be beneficial in this regard. The use of rapid penside tests as well as new technologies on mobile handheld devices in disease response programmes should be integrated for emergency response especially in inaccessible and distant locations to improve disease reporting and minimize communication gaps between farmers and designated veterinary authorities (Aanensen *et al.,* 2009; FAO, 2013).

Although a few farmers (27 %) assumed that the roaming of pigs, visits of other farmers and other factors are responsible for infection of their farms, the majority (68 %) of the surveyed population were not sure of possible causes of outbreaks, indicating that awareness of ASF is poor among the small-scale farmers. Lack of basic biosecurity measures, between and within-farm movements of pigs and free-range/scavenging pig production have been associated with local level spread of ASF in endemic areas especially where smallholder farmers lack awareness of ASF transmission dynamics (Costard *et al.,* 2009). Finally, approximately 94 % of the farmers who lost their pigs to ASF had restocked with or without the observation of the minimum rest period (≥ 40 days up to a maximum quarantine period of six years) for farms post-infection and these actions have huge implications for re-infection or spread of infection to new locations for locations where the soft tick is present (EC, 2002; FAO, 2009; Boinas *et al.,* 2011). We observed that farmers restocked whenever it was convenient for them. Plowright showed in East Africa that pig premises that were neither cleaned nor disinfected were safe restocking 5 but not 3 days after the last pig had died (Plowright *et al*., 1994) an ideology that should be cautiously disseminated to pig farmers.

2.4.1.4 Other socio-anthropogenic and ecologic factors

In Nama, a sub-county of Mukono, the responsible veterinary officer observed and reported a very predictable pattern of outbreaks associated with the dry period months

of June to August, and December to February. In Uganda, these seasons are usually accompanied by scarcity of water in the parks and game reserves; roaming and scavenging pig populations usually move towards and drink from the Ssezibwa River where wild pigs from the Mabira Forest Reserve periphery wallow regularly (Kiryabwire, personal communication). This situation results in unrestricted interactions at the wildlife-domestic animal interface and is usually followed by outbreaks. Jori and colleagues (2009) previously established a similar pattern of disease transmission for foot-and-mouth disease (FMD) at such an interface in ruminant populations in the Kruger National Park.

Since a large proportion of farmers (68 %) were not sure if they had infected farms in the vicinity and unrestricted movements of farmers, scavenging pigs continued unabated in the presence of these outbreaks increasing spread and subsequent outbreaks. Costard *et al.,* (2009) have encouraged the involvement of farmers in policy formulation and development of animal health regulations that minimise the transmission of ASF, if benefits and compliance are to be achieved. ASF has impacted negatively on pig production and affected the livelihoods of pig farmers in Uganda. Muwonge *et al.*, (2012) reported that more pigs were slaughtered during outbreaks regardless of prior testing as a way of minimising losses associated with ASF. The respondents in this study confirmed such losses but only a few (≈1 %) were willing to abandon pig production while the majority (94 %) were resilient and opted for pig farming again. The establishment of good training on basic, community-based and applicable biosecurity will benefit the farmers together with assistance from the government in supply of disease-free stock to farms that experienced outbreaks. It is possible to have free-zones of uninfected pigs even within the endemic zone through

compartmentalization and Costard *et al.*, (2009) previously elaborated on such secure farms.

2.4.2 Conclusions

Within-farm and community-based biosecurity will be important factors for achieving control of ASF in Uganda. Though farmers claimed to have implemented some forms of biosecurity, our assessment revealed that the measures in place are either ineffective or serve as drivers of infection. The adherence to the basic principles of biosecurity and making conscious efforts to avoid the identified associated risk factors and drivers of infection are necessary to improve pig health in Uganda. A high degree of biosecurity sensitisation should be undertaken with the farmers, especially those that hope to become commercial in the trade. The use of veterinary extension services for training and inclusion of social anthropologists, and human behaviourists in the planning and execution of animal health programmes in East Africa is crucial for achieving compliance and reducing risk.

3 CHAPTER THREE

3.1 Introduction

The second study involved using serology and molecular assays to quantify the prevalence of ASF from field samples and identify whether the involved virus genotypes were re-circulating or new genotypes had been introduced in the various geographical regions of Uganda where outbreaks had or had not been reported. It is anticipated that the outputs from this study will improve the policy and decision-making processes in relation to diagnosis of ASF and response to outbreaks and also offer important guidance in formulation of ASF control programs and studies in Uganda.

3.2 Materials and methods

We conducted a cross-sectional survey in seven districts of Uganda namely Pallisa, Lira, Abim, Nebbi, Kabarole, Kibaale, and Mukono from December 2012 to April 2013 (Fig 2-1). These locations were purposely selected because they ensured wide geographic representation of Uganda, had reported outbreaks in recent past and were potentially high-risk locations associated with ASF epidemiology, as defined in sub-section 2.2.1, Chapter 2.

3.2.1 Sample size determination

Sample size was calculated using Epi Info® version 6 based on the exact binomial distribution. We predicted that 193 pig farms would need to be sampled for an estimated 50 % prevalence at 95 % confidence with 10 % precision and a design effect of 2 to account for clustering within districts (Fosgate, 2009). For serology, in order to cater for equal representations in the population to be sampled, 28 pigs from each of the previously defined farms were selected per district hence a total of 193 sera were collected. A 4 ml sample of blood was drawn from the jugular vein of pigs that were

restrained manually (Fig 3-1) and collected in non-heparinized vacutainers. These were centrifuged at 2000 rpm for 15 min and sera were collected in duplicate and stored in well-labelled cryogenic vials at the Animal Virology Unit in NaLIRRI. The sera were then exported to the Transboundary Animal Diseases Programme, Onderstepoort Veterinary Institute, South Africa and stored at 4^{o}C. Serological analysis was performed in the laboratory for all the samples using a blocking enzymatic immunoassay (Blocking ELISA) kit whose mode of action involved an antigen being fixed on a solid support (polystyrene plate) and if a sample serum contained specific antibodies against the virus, they would bind to the antigen adsorbed to the plate while if the serum sample did not contain specific antibodies they would not bind the antigen. If a specific monoclonal antibody (MAb) was added against the viral antigen coated to the plate (conjugated with peroxidase), it would compete with the antibodies of the serum. If the serum samples contained specific antibodies, they would not permit binding of the labelled MAb to the antigen whereas if it did not contain specific antibodies the MAb would bind to the antigen on the plate. After the plate is washed to eliminate all non-fixed material from the plate, the presence or absence of labelled MAb can be detected by adding specific substrate that in presence of the peroxidase develops a colorimetric reaction. The antigen coated to the plate in the immunoassay kit consisted of purified virus protein 73 (VP73), which is the major structural protein from the ASFV and the most antigenic one (Ingezim PPA Compac, Ingenasa Spain).

For the molecular assays, we made an assumption that sampling would be from a large population and that a simple random sampling design would suffice. Using the freedom of disease method we estimated that 14 dead pigs were needed for tissue collection per district for 20 % sensitivity of the virus molecular detection test, for a district with a pig population ranging from 450 to over 100,000 pigs (Fosgate, 2009). Therefore, 98 tissue

samples were required from the seven districts. These were collected from the described farm's slaughter place, abattoir or obtained post-mortem from those farms where dead pigs were found at the time of sample collection. Within the districts, sub-counties served as the primary sampling units, and villages represented the secondary sampling units. These were selected randomly using a multi-stage sampling approach.

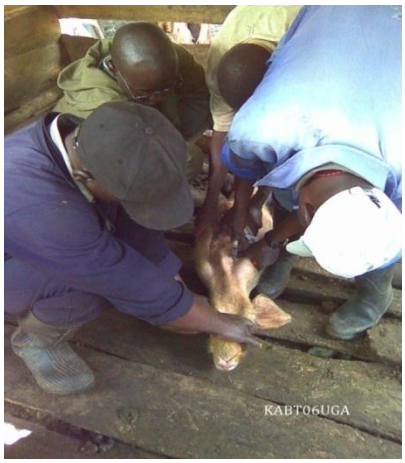

Figure 3-1: NaLIRRI scientists drawing blood from left jugular vein of a manually restrained pig in Kabarole District

3.3 Serological experiments

A total of six plates were used to test the sera (See appendix). Validation of the test for each plate was considered binding when the optical density (OD) of the negative control (NC) was at least 4 times higher than the OD of the positive control (PC). Known positive and negative ASF controls were included for each plate. Two OD readings were obtained and the mean OD was recorded for each sample. The positive and negative cut-off points were calculated using the following formulae respectively: Positive cut-off= NC-[(NC-PC) X 0.5], Negative cut-off= NC-[(NC-PC) X 0.4], where NC corresponds to the OD of the negative control serum and PC corresponds to the OD of the positive control serum. Serum samples with an OD lower than the PC were considered positive to ASFV antibodies and serum samples with an OD higher the NC

were considered negative to ASFV antibodies. Serum samples with OD values between both cut offs were considered doubtful, and this study did not have any in that category.

3.4 Molecular assays

3.4.1 Extraction and genomic amplification of the viral DNA

Tissues from the field were collected into well-labelled, tight-sealed, 10ml Falcon tubes and were stored at 4^{o}C, then transported to the Animal Virology Unit (AVU) in NaLIRRI, from where they were exported to the Transboundary Animal Diseases Program, Onderstepoort Veterinary Institute, South Africa, under UN guidelines (Table 3:1).

Table 3-1 Summary of the tissue type collected and District of origin of the field samples from 59 domestic pigs in seven districts of Uganda, 2012-2013. (Total collected 78, individual pooled 10)

District	Sub-county	Village	Lab Id	Sample ID	Sample Type	Source
Pallisa	Apopong	Kachip	1	01	Liver	Home Slaughter
	Agule	Okume	2	02	Liver	Home Slaughter
	Pallisa	Rarakoi	3	03	Blood Clot	Home Slaughter
	Agule	Hospital Ward	4	04	Liver	Home Slaughter
	Gogonya	Aujabule	5	05	Liver	Home Slaughter
	Apopong	Okorotok	6	06	Liver	Home Slaughter
	Apopong	Okorotok	7	07	Liver	Abattoir
	Apopong	Okorotok	8	08	Liver	Home Slaughter
	Apopong	Kasabio	9	09	Liver	Home Slaughter
	Apopong	Kasabio	10	10	Kidney	Dead Pig
Lira	Ojwina	Wigweng	11	1-2	Kidney	Home Slaughter
	Ojwina	Wigweng	12	2-2	Kidney	Home Slaughter
	Ojwina	Wigweng	13	3-2	Kidney, Mesenteric LN	Home Slaughter
	Ojwina	Wigweng	14	4-2	Kidney	Home Slaughter
	Ojwina	Wigweng	15	5-2	Kidney	Home Slaughter
	Ojwina	Wigweng	16	6-2	Kidney	Home Slaughter
	Agali	Alipot	17	7-2	Kidney	Home Slaughter
	Agali	Alipot	18	8-2	Kidney	Home Slaughter
	Agali	Alipot	19	9-2A	Kidney	Home Slaughter
	Agali	Alipot	20	9-2B	Liver	Home Slaughter
	Agali	Alipot	21	10-2	Kidney	Home Slaughter
	Agali	Alipot	22	11-2	Kidney	Home Slaughter
	Agali	Alipot	23	12-2	Kidney	Home Slaughter

District	Sub-county	Village	Lab Id	Sample ID	Sample Type	Source
	Agali	Alipot	24	13-2	Spleen, Mesenteric LN	Home Slaughter
	Agali	Alipot	25	14-2A	Liver	Home Slaughter
	Agali	Alipot	26	14-2B	Mesenteric LN	Home Slaughter
	Amach	Akuli	27	1-3	Liver Kidney Heart	Dead Pig
	Amach	Akuli	28	2-3	Kidney	Home Slaughter
Abim	AbimTc	Yenglemi East	29	3-3	Lung Mesenteric LN	Abattoir
	AbimTc	Oyaro Cell	30	4-3	Kidney	Home Slaughter
Nebbi	Nyaravur	Angal	31	1-4	Kidney Liver Heart	Abattoir
	Nyaravur	Angal	32	2-4	Muscle Tissue	Abattoir
	Parombo	Parwo	33	3-4	Heart, Kidney, Liver, Lymph Node	Abattoir
	Parombo	Parwo	34	4-4	Muscle Tissue	Abattoir
	Parombo	Alegu East	35	5-4	Hard Tick	Live Pig
	Nyaravur	Alegu East	36	7-4	Spleen	Dead Pig
	Nyaravur	Alegu East	57	8-4	Spleen	Abattoir
Kabarole	Busuro	Rwengaju	37	1-5	Kidney	Abattoir
	Busuro	Akibasi	38	2-5A	Kidney Heart Liver	Abattoir
	Busuro	Akibasi	39	2-5B	Spleen	Abattoir
	Hakibale	Hakibale	40	3-5	Kidney Liver Lymph Node	Abattoir
	Hakibale	Nsoro	41	5-5	Hard Tick	Abattoir
	Nyaravur	Angal	42	14-4	Muscle	Home Slaughter
	Hakibale	Kyaitamba A	43	6-5	Liver Kidney Lymph Node	Abattoir
	Hakibale	Kyansimbi	51	9-5	Lice	Survivor Pig
Kibaale	Kyanaisoge	Kisunga	44	1-6	Liver Heart Kidney Lymph Node	Abattoir
	Muhooro	Muhooro TC	45	2-6	Liver	Abattoir
	Muhoro	Muhooro TC	46	3-6	Liver	Abattoir
	Muhoro	Karusigwa	47	4-6	Lymph Node	Abattoir
	Muhoro	Karusigwa	48	5-6	Lymph Node	Abattoir
	Muhoro	Karusigwa	49	6-6	Lymph Node	Abattoir
	Muhoro	Karusigwa	50	7-6	Spleen	Abattoir
	Muhoro	Karusigwa	52	9-6	Spleen	Abattoir
	Muhoro	Nyamitti C	54	10-6	Spleen	Abattoir
	Muhoro	Nyamitti C	55	11-6	Spleen	Abattoir
	Muhoro	Nyamitti C	56	12-6	Lymph Node	Abattoir
	Muhoro	Nyamitti C	59	13-6	Liver	Abattoir
	Muhoro	Karusigwa	53	14-6	Tick	Pig
Mukono	Nama	Namawojjolo	58	1-7	Liver	Abattoir

3.4.2 DNA extraction

DNA extraction from 78 tissues, with 19 of these being prepared from pooled tissues from the same animal (as detailed in Table 3.1 above) was performed using Roche High Pure PCR Template Preparation Kit (version 16.0). The 59 purified DNA templates

were screened by conventional PCR using OIE prescribed forward primer ASF-1 (ATGGATACCGAGGGAATAGC) and the antisense ASF-2 primer (CTTACCGATGAAAATGATAC) that target a 278bp fragment of the ASF *p72* gene. Products were run on a 1 % Agarose MP gel (Roche) stained with ethidium bromide and visualised under UV. Positive amplicons were heat inactivated at 60^{o}C for 1 hour prior to transporting them to Mammal Research Institute, University of Pretoria for multi locus typing and sequencing.

3.4.3 Genomic amplification

Forward and reverse primers for four gene regions of the ASF genome were used for multi locus typing. All reactions were performed using a touchdown PCR thermal cycling approach in combination with Biotools*Taq* polymerase (1U/reaction). The four gene regions were the *p54* gene, *p72* gene, the central variable region (CVR) of the *9RL*ORF and *TK* gene. Each primer setwas assigned a single letter code, *viz.*: [A] PPA89 + PPA722 (Gallardo *et al*. 2009) – targets *p54,* [C] CVR-FLF + CVR-FLR (Bastos *et al*. 2004) – targets CVR of the *9RL* ORF, [P] *p72*-U + *p72*-D (Bastos *et al*. 2003) – targets C-terminal end of *p72*and [T] *TK*-1 + *TK*-Rev (Fasina *et al*. in prep) – targets Thymidine kinase gene. Reactions were performed in a final volume of 50 µl containing each of the primers at a final concentration of 0.4 µM, in the presence of 1U of Biotools *Taq* polymerase. DNA templates were assigned a unique number as follows: 1. 10, 2. 7/4, 3. 3/6, 4. 6/6, 5. 9/6, 6. 1/7, 7. 13/6, 8. Negative control, and 4 µl of template was added to the A, C and P reactions, whilst 3 µl was added to each 'T' reaction tube. A touch-down PCR was performed on a gradient thermal cycler, with the following annealing temperatures and number of cycles: [A] 57°C x 2; 56°C x 3; 55°C x 35, [C] 54°C x 2; 53°C x 3; 52°C x 35 , [P] 52°C x 2; 51°C x 3; 50°C x 35 and [T]

49°C x 2; 48°C x 3; 47°C x 35 . All annealing steps were preceded by denaturation at 96°C for 12s, and followed by an extension/elongation step at 70°C for 1 minute.

3.4.4 Purification and cycle sequencing

All PCR products were purified using Roche High Pure PCR Purification Kit. The purified product was eluted in a final volume of 50 µl of 1:1 Elution buffer: ddH_2O. All *p72* and CVR amplicons 1, 2, 5, 6 and 7 were sequenced with each of the PCR primers in separate reactions at an annealing temperature of 50°C and 52°C, respectively. For the *TK* and *p54* amplicons, 7 and 3 purified products respectively were sequenced with each of the PCR primers in separate reactions. For the *TK* gene these were samples: 1-7 and for the *p54* gene it was samples 2, 6 and 7. The *TK* reactions were cycle sequenced at an annealing temperature of 48°C while the *p54* reactions were sequenced at an annealing temperature of 54°C. All products were ethanol-precipitated using sodium acetate and submitted to the FABI DNA sequencing facility.

3.5 Results and analysis

3.5.1 Serological assay

Only six out of 25 sera from Abim district tested positive while sera from other locations were negative giving an overall prevalence of 3.1 %, and a regional prevalence of 24% (Table 3-2).

Table 3-2.Serology results of samples collected from the seven districts of Uganda

District	Samples positive (%)	Minimum optical density	Maximum optical density	Samples negative (%)	Minimum optical density	Maximum optical density
Pallisa	0 (0)	-	-	28 (100)	1.876	2.509
Lira	0 (0)	-	-	28 (100)	1.901	2.349
Abim	6 (24)	0.058	0.739	19 (76)	1.749	2.254
Nebbi	0 (0)	-	-	28 (100)	1.844	2.489
Kabarole	0 (0)	-	-	28 (100)	1.815	2.519
Kibaale	0 (0)	-	-	28 (100)	1.9	2.736
Mukono	0 (0)	-	-	28 (100)	1.905	2.55
Total	**6 (3.1)**			**187 (96.9)**		

3.5.2 Molecular assays

3.5.2.1 Agarose gel electrophoresis of p72-PCR products using OIE screening primers

A total of seven amplicons were positive out of the 59 DNA extracts (Fig. 3-2).

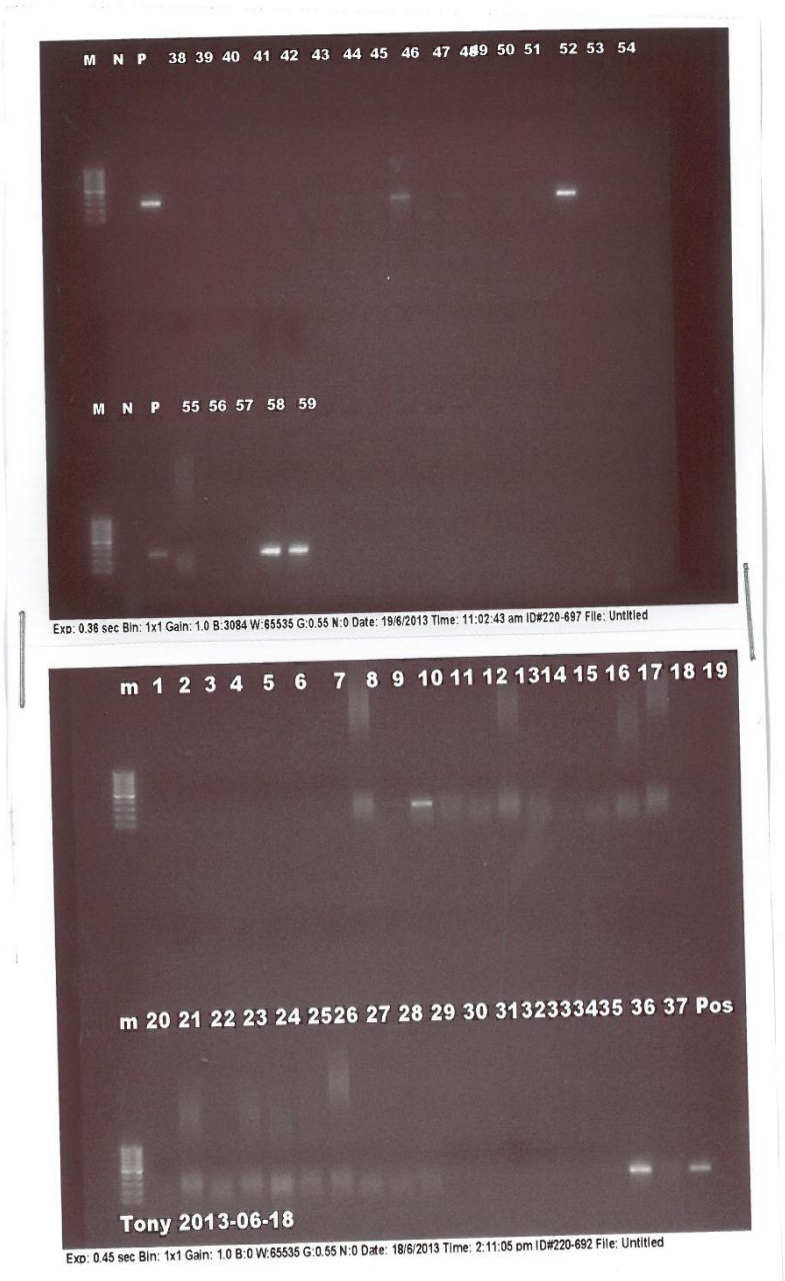

Figure 3-2Agarose gel electrophoresis of *p72*-PCR products amplified with P1 and P2 OIE *p72* screening primers. Lane m: 100 bp ladder; lane N: Negative control; lane P: Positive control; lanes 10, 36, 46, 48, 52, 58 and 59; Positive amplicons.

3.5.2.2 Multi locus typing

Agarose gel bands for positive cycle sequenced products were evident in lanes A2, A6, A7, C1, C2, C5, C6, C7, P1, P2, P5, P6, P7, T5 and T6 (Fig 3-3) and T1, T2, T3, T4 and T7 (Fig 3-4)

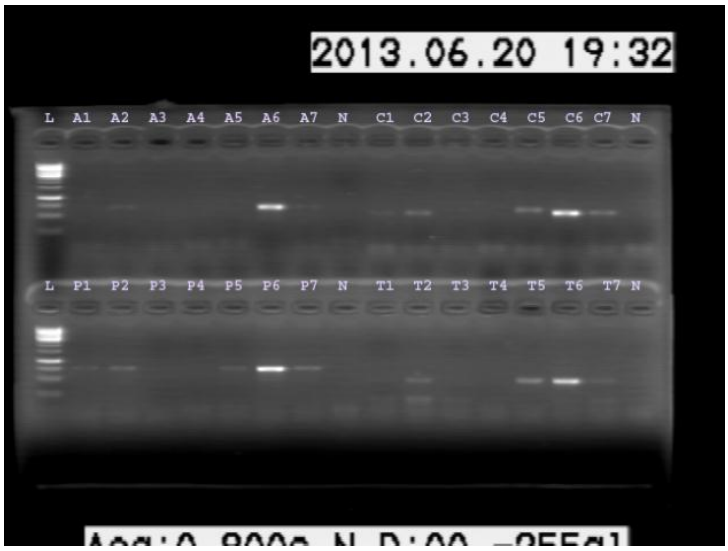

Figure 3-3: Agarose gel electrophoresis of *p54* [A], *CVR-ORF* [C], *p72* [P] and *TK* [T] gene products. Lane L: 100 bp ladder; lane N: Negative control; lanes A2, A6, A7, C1, C2, C5, C6, C7, P1, P2, P5, P6, P7, T5 and T6; Positive products

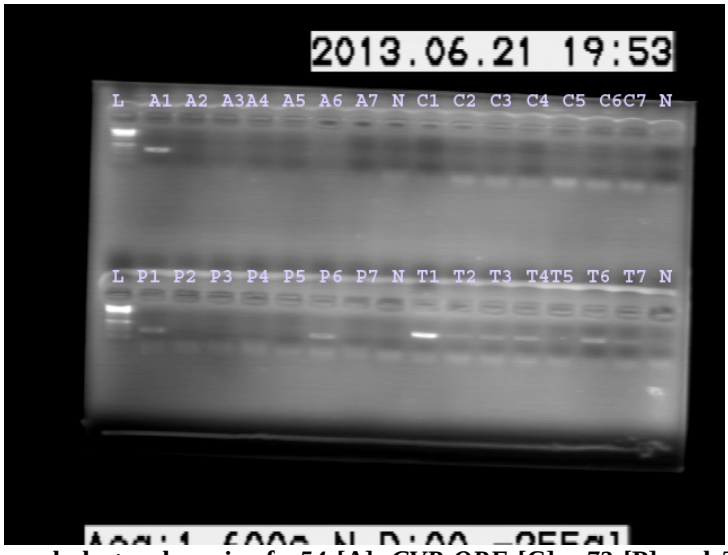

Figure 3-4: Agarose gel electrophoresis of *p54* [A], *CVR-ORF* [C], *p72* [P] and *TK* [T] gene cycle sequenced products using modified reaction conditions and demonstrating improved *TK* gene amplification. Lane L: 100 bp ladder; lane N: Negative control; lanes T1, T2, T3, T4 and T7; Positive products.

3.5.2.3 Phylogenetic analyses

Phylogenic inference of ASF DNA sequences was carried out using analyses specific for the different gene regions. Published sequences were added from Genbank as shown in Section 5.2 (See appendix).

3.5.2.4 P72 Gene

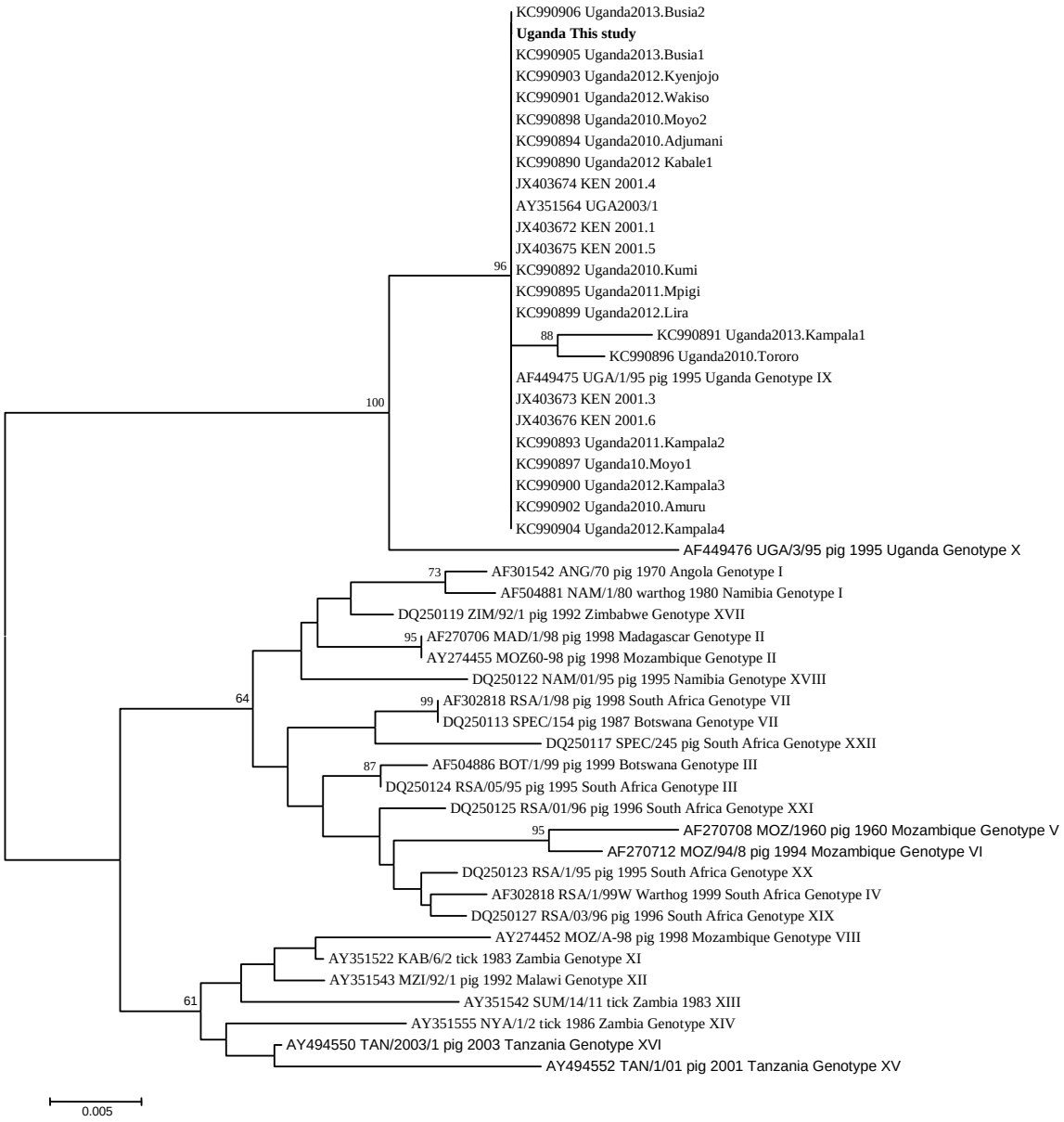

Figure 3-5: Neighbor joining *p72* gene tree

3.5.2.5 CVR-ORF tetramer alignment

Figure 3-6: Tetramer alignment of the CVR of the *9RL*ORF (C1-C7, indicated in bold are derived from this study)

3.5.2.6 P54 gene

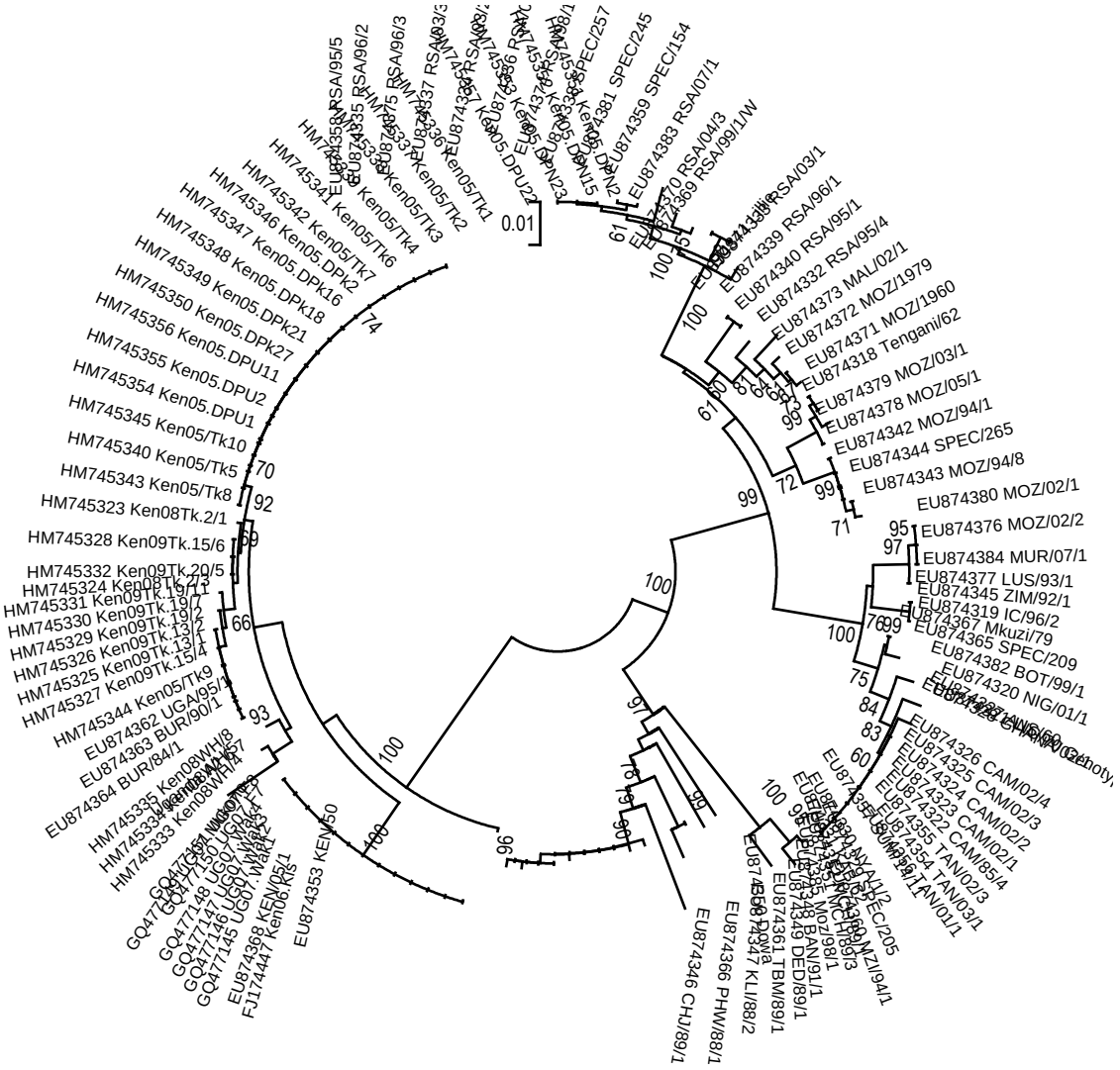

Figure 3-7: Neigbor Joining *p54* tree

3.5.2.7 TK gene

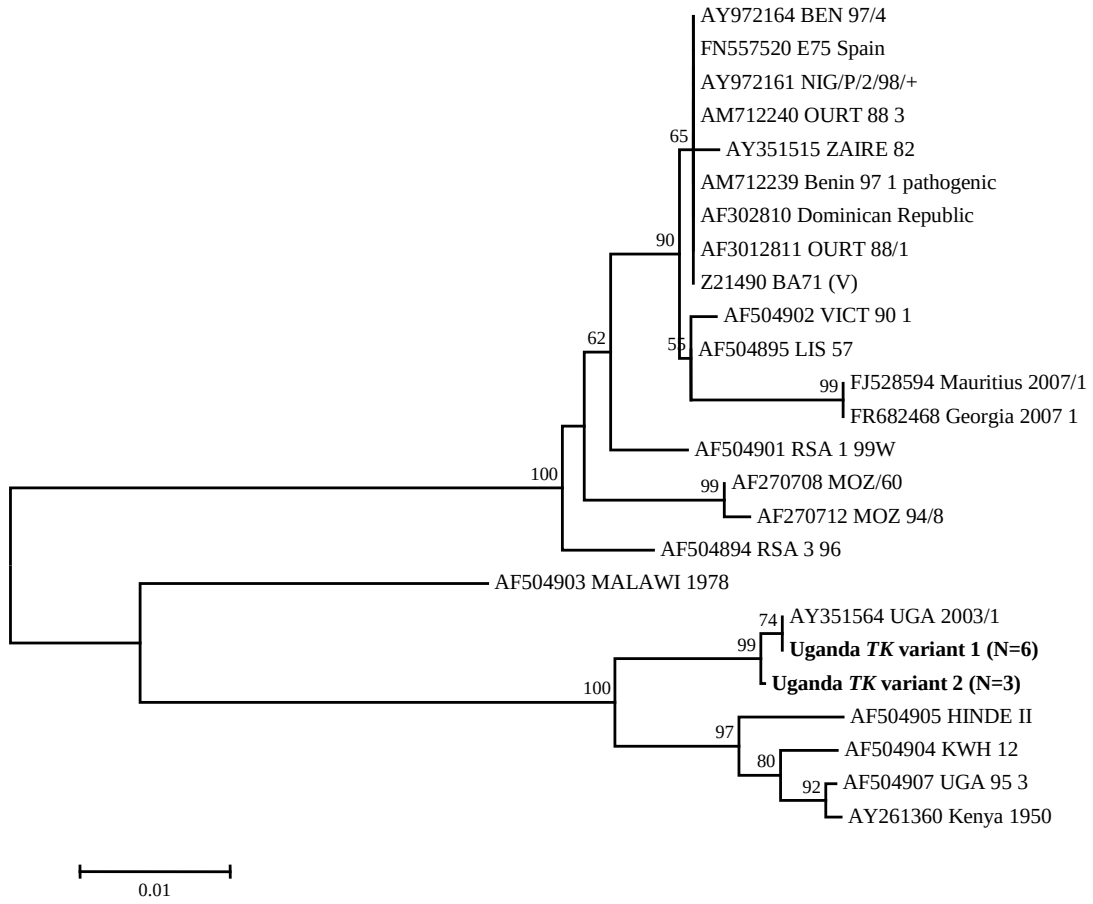

Figure 3-8 Neighbor-joining *TK* gene tree. Viruses in bold indicate those characterised in this study.

TK gene affirmation

From the PCR results of the four gene regions as shown in sub section 3.5.2.2, the *TK* primers produced more amplicons of the expected size (7 in total), of which six were confirmed by nucleotide sequencing to correspond to the viral genome target. This finding of superior ASF genome detection impelled the re-testing of all 59 DNA extracts previously assigned ASF-positive status with the OIE recommended *p72* gene primers, with the *TK*-1 and *TK*-Rev primers. Remarkably 3 more positives were identified amongst these. Following nucleotide sequence alignment, two *TK* gene variants were detected and their relationship to available homologous data is shown in Fig 3.9.

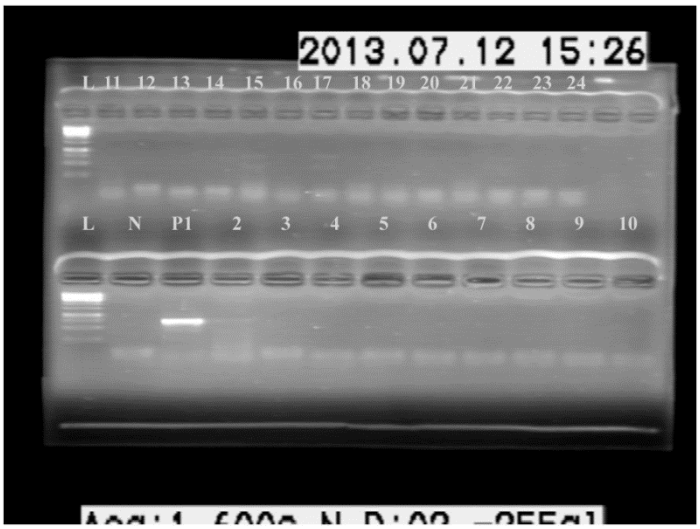

Figure 3-9: Agarose gel electrophoresis of *TK* gene-PCR products amplified with *TK*-1 + *TK*-Rev primers. Lane L: 100 bp ladder; lane N: Negative control; lane P1: Positive control; lanes 2, 15 and 17; *TK* gene positive amplicons.

3.6 Discussion

This study evaluated the impact of serology in the determination of the prevalence of ASF from field samples, and the expediency of molecular tools in the investigation of ASFV genomes in the various geographical regions of Uganda where outbreaks had or had not been reported.

3.6.1 Serological patterns in on-going and post-outbreak situations in Uganda

Baseline prevalence data from the regions described in the study sites were collected, since there were no published results of seroprevalence from any of them. Six out of 25 sera collected from Abim district tested positive while all sera from other locations were negative indicating an overall prevalence of 3.1 %, and a regional prevalence of 24 %. No serum samples were positive where current outbreaks were observed (Table 3-2). In a similar study, all sera collected from clinically sick pigs that were tested using the prescribed OIE serological tests were negative (Gallardo *et al*., 2011). Perez-Filguera *et al*., (2006) noted that when they used both the recombinant and conventional ELISAs

on the *p30r* protein, variable rates of sensitivity and specificity with the African samples were observed, especially regarding their geographical origin. They further highlighted that if those rates were associated with antigenic variations among isolates, it would be necessary to produce additional versions of *p30r* from those ASFV serotypes more distant to genotype I and include them in the antigen preparation. Gallardo *et al*., (2011) attributed this unexpectedly low seropositive response with east African sera, especially when the OIE-prescribed methods are used, to the immunogenetics of the indigenous pig populations and not the polymorphisms in immunodominant viral antigens. Since ASF is a rapidly fatal disease, it is possible that the infected domestic pigs quickly die before the development of antibodies, are culled or are sold off to the market, a practice that is rife amongst smallholder pig farmers in Uganda. Muwonge *et al.,* (2012) also noticed that farmers in Mubende slaughtered a huge number of pigs during an outbreak to minimise the losses from pig deaths. This would explain the lower antibody titres detected implying serology may not be a good indicator of ASF status in an active outbreak. Limited serological assays conducted for ASF in other locations within Uganda recovered a prevalence of 2.1 % and 0.2 % (Björnheden, 2011; Muwonge *et al.,* 2012), whilst in Nigeria levels (9 %) were similarly low (Fasina *et al.,* 2010). The genetic analysis and virology of samples taken from the same sites as serology were positive even where all sera were negative (Fig 3-10).

In this study, we found that if serology only is used as a definitive diagnostic tool for ASF, there is approximately an 88 % chance of missing a positive sample therefore ASF antibody detection may not be a good indication of the field situation during an on-going outbreak or in the immediate post-outbreak situation.

At the time of sample collection for this study, some sampled sites had on-going outbreaks and quarantine. It is important to note that in Abim district where positive

serology was obtained, formal production systems for pigs were low to inexistent in comparison to other districts and the farmers merely identified their pigs during marketing or slaughter. Whether this played a role in the serological patterns and morbidity is not clear. For all the other districts, the majority of farmers housed their pigs, a factor thought to reduce the morbidity of disease. However this did not fully clarify why antibody was not detected in some areas with on-going outbreaks, even after the postulated 14 days post infection. It is possible that the serological test may not be sufficiently specific for the ASF antigens circulating in Uganda or those pigs do not produce antibodies like recently reported in Kenya (Gallardo *et al.*, 2012) warranting future studies to confirm.

In Uganda, the twin-situation of on-going outbreaks and disease endemicity makes it difficult to disentangle endemic spread and new infections. A similar survey conducted in Senegal showed that the presence of antibodies to ASF was only an indication of a previous encounter of ASF at a point in time, and not an indicator of current infection (Etter *et al.*, 2011). Our results concur with this, although it does not inform the period of occurrence of the past outbreak. In their study, Hutchings and Ferris, 2006, suggested that for new introductions of disease it was better to detect the virus by serology, and to base the diagnosis in endemic areas on antibody tests. This may, however, prove difficult in Uganda where the epidemiological situation is complex and a clear distinction between endemic and new infections cannot be drawn. Hutchings and Ferris, 2006, also suggested that ELISA alone should not be used as a diagnostic test because it may not be able to detect low concentrations of viruses, especially from poor quality diagnostic material but recommended that a more sensitive method to test the virus should instead be incorporated for routine diagnosis. This would be beneficial if it considered that clinical samples usually differ in their concentrations, and the viral loads

within individual pigs are also different. If the results from serology alone are used, there is a likelihood of inadvertent spread of disease as animal experts and officials are guided by these results when implementing control and mitigation programs. Therefore, serology alone is unlikely to be adequate and more sensitive molecular techniques in combination with serology are emphasized in the definitive diagnosis of ASF.

3.6.2 Molecular epidemiology of ASF in Uganda

Four gene regions were used for detection of viruses from domestic pigs in Uganda. The assays produced 41 amplicons, which when sequenced confirmed ASF genome presence in ten domestic pigs (Table 3-3).

Table 3-3: Summary of PCR & sequencing results

Sample Name	Locality	Tissue Type	Sample No.	OIE Diagnostic PCR	[A]	[C]	[P] *P72*-U + *p72*-D	[T] *TK*1 + *TK*-rev
10	Pallisa	Kidney	1	+	_	+	_	+
36	Nebbi	Spleen	7/4	+	+	+	+	+
46	Kibaale	Liver	3/6	+	_	_	_	+
48	Kibaale	Mesenteric lymph node	5/6	+	_	_	_	_
52	Kibaale	Spleen	9/6	+	_	+	+	+
58	Mukono	Liver	1/7(Wak)	+	+	+	+	+
59	Kibaale	Liver	13/6 (Kab)	+	+	+	+	+
2a	Nebbi	Spleen	8/4	_	_	_	_	+
15	Abim	Lung, mesenteric lymph node	3/3	_	_	_	_	+
17	Lira	Liver, Kidney, heart	1/3	_	_	_	_	+

The *p72* and *p54* gene regions confirmed that all viruses belonged to *p72* genotype IX (Fig.3-5& 3-7) and the majority of the variants were identical to one of the 1995 Ugandan viruses. Alignment of tetramers of the CVR-9ORF gene region (Fig. 3-6) recovered 3 different CVR variants for the six positive amplicons of which, two had 23 tetramers, two had 24 tetramers and one (C5) had 29 tetramers (Fig. 3.6). The initial screening with the *p72* gene revealed the presence of ASF viruses in four districts namely Pallisa, Nebbi, Kibaale and Mukono. At this stage, no viruses were detected in

Lira, Abim and Kabarole. Fernández-Pinero *et al*., (2012) noted that the real-time PCR procedure offered good sensitivity and specificity rates though with the analysis of weak ASFV-positive samples the robustness of the method was decreased. This could partly explain the results from the initial screening PCR we carried out because we used field samples that obviously were different in concentrations.

3.6.2.1 Phylogeography of the viruses

The *TK* gene performed better by amplifying 16 targets, 4 of which were new detections earlier missed by the *p72* gene (Fig. 3-10). Reasons for this include a possible change in the genome diversity and strain variability of the ASFV or more sensitivity offered by the *TK* gene. Fernández-Pinero *et al*., (2012) further noted that more than 10% of positive samples were not detected when reference TaqMan PCR was used in comparison to the new UPL PCR. This finding supports our result that the reference p72 PCR was not able to detect all viruses but repeatability tests are needed to confirm the *TK* gene performance. Two sequence variants were recovered with *TK* within which a single nucleotide mutation was detected which results in a synonymous amino acid (aa) at that site. Also important to mention is that there is a premature stop codon resulting in a *TK* protein 185 aa in length instead of 196 aa.

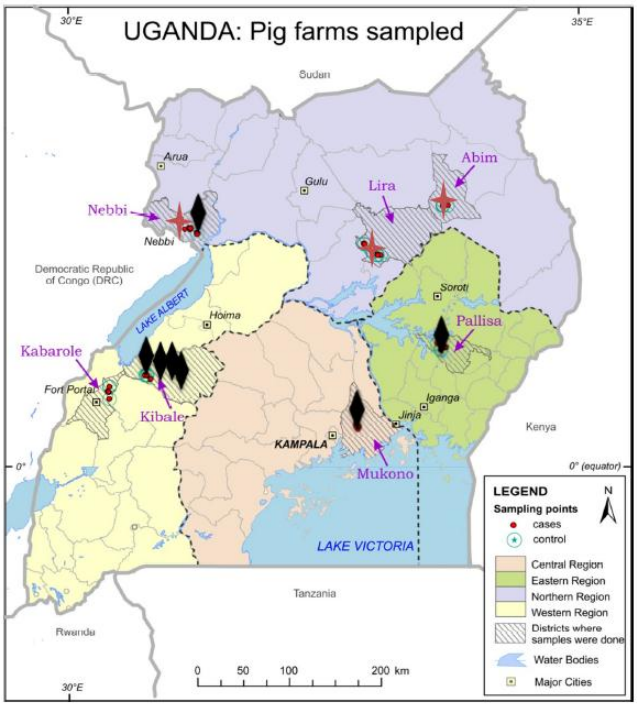

Figure 3-10: Map of Uganda showing positive characterised strains of ASFV 2012-2013. The black diamond indicates strains detected by the OIE *p72* gene diagnostic assay, and the red star indicates the localities of the three additional, sequence-confirmed ASF cases detected by *TK* gene.

In a recent phylogenetic study of ASF in Uganda, Atuhaire *et al*., 2013b, detected 21 viruses out of the 30 outbreaks saying that the 9 undetected could have been due to another aetiology since PCR is highly sensitive. We found that the two *p72* gene assays used in this study were not able to detect three variants from Abim, Lira and Nebbi districts. The *TK* gene detected these viruses and also detected two more which were positive for the *p72* gene and negative with the *p54* gene primers. We hypothesize that the viral load in the field samples could be low in concentration hence the low levels of amplification, or that viruses with different virulence could be co-circulating. Since the *TK* gene is associated with virulence, we think that it is useful in outbreak situations for detecting variant strains. Justification needs to be sought as to why the *p72* gene could not detect the viruses where the *TK* did, because this has grave effects for viral surveillance and control. Where control programs are designed for areas where outbreaks were identified with *p72* gene, there is a possibility of leaving out those with

the undetectable viruses, hence carrying on the outbreaks and wasting resources. The fact that ASF may be under-diagnosed creates a big challenge in control, since such viruses will be transmitted inadvertently to areas where no control is being implemented. More studies are needed to confirm the possibility of changes in the p72 gene and the reliability of the *TK* region as a good region for diagnosis. This could be done by additional analysis of the *TK* gene positive samples. In their study, Atuhaire *et al*., 2013 did not find any reported outbreaks in the North Eastern region. We found positive samples on serology in Abim, and also a virus strain from tissue using the *TK* gene region further showing the advantage of the *TK* gene in diagnosis. The CVR region of the *9RL* ORF detected one virus sequence from Pallisa district, confirming virus presence in one district more than what was detected by the *p72* and *p54* gene regions, which both detected viruses in just three districts (Lira, Kibaale and Mukono).

3.6.3 Conclusion

The molecular study confirms the endemicity of ASF in Uganda and shows the superiority of molecular assays over serological ones. Repeatability tests are needed to confirm the *TK* gene performance as a good region for diagnosis if the *TK* gene sequencing and characterisation should be included in routine ASF diagnosis in EA. Future tests should target proteins and antigens that can be identified in early responses to ASFV infection which would be beneficial in reducing the time required for diagnosis. There is a need to find out how many repeat outbreaks are sequels from re-infection from survivor pigs and identify any association. The immunological basis for such survivor animals should be sought and analysed. There is need to establish re-stocking programs that ensure availability of virus-free pigs with guidelines for biosecurity on pig farms, and of the pig farmers.

3.7 Ethical clearance

This project passed all ethical clearance of the Ugandan government and was approved by the Faculty Research Committee with approval number: V052/12“Molecular and Serological Epidemiology of African Swine Fever in Domestic Pigs in Uganda”. All samples involved in the work were transported to the OIE Reference laboratory for ASF, Transboundary Animal Disease Programme-Onderstepoort Veterinary Institute, South Africa in compliance with the UN standards for transport of infectious material (UN2900; http://www.who.int/ihr/training/laboratory_quality/5_c_annex_G_cd_rom_sample_transport_info.pdf)

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5 APPENDIX

5.1 Laboratory results showing Optical Densities of 190 sera from 7 districts in Uganda, 2012-2013.**Replaced sample, initial sample vial was empty, Bold ODs show positive sera*

Plate validity	sample ID	OD 1	OD 2	Mean OD	Positive cut off/ plate	Negative cut off/plate
I	**Positive**	0.085	0.086	**0.0855**	**1.0745**	**1.2723**
24.135	**negative**	2.075	2.052	**2.0635**		
	1	2.261	2.476	2.369		
	2	2.19	2.506	2.348		
	3	2.137	2.548	2.343		
	4	2.166	2.067	2.117		
	5	2.1	2.048	2.074		
	6	1.964	1.985	1.975		
	7	1.903	1.848	1.876		
	8	2.068	2.073	2.071		
	9	1.993	2.03	2.012		
	10	1.927	2.004	1.966		
	11	2.124	2.241	2.183		
	12	2.104	2.108	2.106		
	13	2.009	1.959	1.984		
	14	2.026	2.119	2.073		
	15	2.148	2.22	2.184		
	16	2.449	2.569	2.509		
	17	2.375	2.405	2.39		
	18	2.046	2	2.023		
	19	2.018	2.016	2.017		
	20	2.004	1.986	1.995		
	21	2.183	2.168	2.176		
	22	2.126	2.203	2.165		
	23	2.202	2.155	2.179		
	24	2.159	2.165	2.162		
	25	2.091	2.058	2.075		
	26	2.095	2.082	2.089		
	27	1.971	1.98	1.976		
	28	1.979	2.006	1.993		
	29	2.028	1.968	1.998		
	30	2.179	2.174	2.177		
	31	2.123	2.123	2.123		
	32	2.233	2.25	2.242		
	33	1.993	2.03	2.012		
	34	2.13	2.155	2.143		
	35	2.158	2.156	2.157		
	36	2.035	2.034	2.035		
	37	1.909	1.893	1.901		
	38	2.287	2.311	2.299		
	39	2.197	2.242	2.22		
	40	2.073	2.084	2.079		
	41	2.095	2.15	2.123		
	42	2.222	2.309	2.266		
	43	2.119	2.14	2.13		
	44	2.046	2.088	2.067		
	45	2.004	2.007	2.006		
	46	2.089	2.11	2.1		
II	**Positive**	0.093	0.105	0.099	**1.08075**	**1.2771**
20.833	**negative**	2.065	2.06	2.0625		
	47	2.179	2.276	2.228		

	48	2.118	2.069	2.094		
	49	2.337	2.258	2.298		
	50	2.38	2.317	2.349		
	51	2.151	2.1	2.126		
	52	2.162	2.127	2.145		
	53	2.321	2.241	2.281		
	54	2.115	2.159	2.137		
	55	2.072	2.102	2.087		
	56	2.035	2.047	2.041		
	57	2.233	2.244	2.239		
	58	2.212	2.195	2.204		
	59	**0.21**	**0.311**	**0.261**		
	60	2.043	2.063	2.053		
	61	**0.056**	**0.06**	**0.058**		
	62	**0.076**	**0.066**	**0.071**		
	63	**0.7**	**0.778**	**0.739**		
	64	2.259	2.243	2.251		
	65	2.182	2.167	2.175		
	66	2.136	2.132	2.134		
	67	**0.063**	**0.071**	**0.067**		
	68	2.099	2.113	2.106		
	69	2.194	2.313	2.254		
	70	2.094	2.001	2.048		
	71	2.171	2.082	2.127		
	72	1.817	1.748	1.783		
	73	2.223	1.989	2.106		
	74	2.122	2.062	2.092		
	75	1.871	1.831	1.851		
	76	1.806	1.691	1.749		
	77	2.2	2.279	2.24		
	78	2.131	2.183	2.157		
	79	**0.275**	**0.293**	**0.284**		
	80	1.79	1.994	1.892		
	81	2.17	2.233	2.202		
	93*	2.122	2.127	2.125		
	94*	1.873	2.095	1.984		
	95*	1.951	2.104	2.028		
	85	2.053	2.313	2.183		
	86	2.072	2.156	2.114		
	87	2.13	2.204	2.167		
	88	2.071	2.208	2.14		
	89	2.077	2.13	2.104		
	90	2.013	2.056	2.035		
	91	2.235	2.317	2.276		
	92	2.18	2.263	2.222		
III	**Positive**	0.067	0.067	0.067	**1.01625**	**1.2061**
29.336	**negative**	1.974	1.957	1.9655		
	96	2.471	2.507	2.489		
	97	2.353	2.401	2.377		
	98	2.037	2.037	2.037		
	99	2.12	2.093	2.107		
	100	2.107	2.063	2.085		
	101	2.117	2.054	2.086		
	102	2.026	1.994	2.01		
	103	2.107	2.038	2.073		
	104	1.977	1.916	1.947		
	105	1.979	1.935	1.957		
	106	2.066	2.076	2.071		
	107	1.96	1.96	1.96		

	108	1.952	1.956	1.954		
	109	1.964	1.978	1.971		
	110	2.048	2.071	2.06		
	111	2.059	2.051	2.055		
	112	1.858	1.829	1.844		
	113	1.87	1.873	1.872		
	114	2.521	2.516	2.519		
	115	1.961	1.948	1.955		
	116	1.897	1.957	1.927		
	117	1.995	2.015	2.005		
	118	1.893	2.005	1.949		
	119	2.036	2.008	2.022		
	120	1.997	1.994	1.996		
	121	1.887	1.911	1.899		
	122	1.936	1.933	1.935		
	123	1.836	1.818	1.827		
	124	1.844	1.871	1.858		
	125	1.791	1.911	1.851		
	126	1.969	1.95	1.96		
	127	1.888	1.892	1.89		
	128	1.954	1.884	1.919		
	129	1.875	1.881	1.878		
	130	1.951	1.908	1.93		
	131	2.111	2.238	2.175		
	132	1.915	1.897	1.906		
	133	2.059	2.048	2.054		
	134	1.886	1.899	1.893		
	135	1.843	1.856	1.85		
	136	1.819	1.81	1.815		
	137	1.92	1.991	1.956		
	138	2.176	2.186	2.181		
	139	1.919	1.965	1.942		
	140	1.815	1.9	1.858		
	141	1.992	1.979	1.986		
IV	**Positive**	0.079	0.07	0.0745	**1.0825**	**1.2841**
28.06	**negative**	2.113	2.068	2.0905		
	142	2.163	2.122	2.143		
	143	2.21	2.136	2.173		
	144	2.371	2.284	2.328		
	145	2.135	2.111	2.123		
	146	2.077	2.002	2.04		
	147	2.262	2.219	2.241		
	148	2.281	2.303	2.292		
	149	2.116	2.131	2.124		
	150	2.235	2.194	2.215		
	151	2.11	2.049	2.08		
	152	2.127	2.164	2.146		
	153	2.529	2.497	2.513		
	154	2.072	2.075	2.074		
	155	2.013	2.013	2.013		
	156	2.774	2.698	2.736		
	157	2.08	2.094	2.087		
	158	2.073	2.069	2.071		
	159	2.037	2.037	2.037		
	160	2.023	2.123	2.073		
	161	2.098	2.153	2.126		
	162	1.939	1.998	1.969		
	163	2.056	2.092	2.074		
	164	2.145	2.038	2.092		

	165	1.982	1.956	1.969		
	166	1.943	1.912	1.928		
	167	1.973	1.932	1.953		
	168	1.966	1.833	1.9		
	169	2.002	1.947	1.975		
	170	1.996	1.914	1.955		
	171	2.004	1.993	1.999		
	172	2.218	2.22	2.219		
	173	2.042	2.047	2.045		
	174	1.967	2.011	1.989		
	175	2.056	2.044	2.05		
	176	1.954	1.954	1.954		
	177	1.901	1.908	1.905		
	178	2.027	2.009	2.018		
	179	2.058	2.082	2.07		
	180	2.228	2.278	2.253		
	181	2.197	2.191	2.194		
	182	2.064	1.999	2.032		
	183	2.098	2.138	2.118		
	184	2.106	2.181	2.144		
	185	1.884	2.049	1.967		
	186	1.99	2.049	2.02		
	187	2.165	2.212	2.189		
V	**Positive**	0.432	0.387	0.4095	**1.4045**	**1.6035**
5.86	**negative**	2.439	2.36	2.3995		
	188	2.449	2.384	2.417		
	189	2.464	2.417	2.441		
	190	2.561	2.538	2.55		
	191	2.462	2.438	2.45		
	192	2.415	2.446	2.431		
	193	2.278	2.313	2.296		

5.2 Summary of reference ASF virus strains/isolates used in the phylogenetic inference for *p72* gene

Strain name	Country	Year of isolation	*P72*Genbank Accession number	Species of origin	*P72* genotype
Uganda2013.Busia2	Uganda	2013	KC990906	Pig	Genotype IX
			Uganda This study		
Uganda2013.Busia1	Uganda	2013	KC990905	Pig	Genotype IX
Uganda2012.Kyenjojo	Uganda	2012	KC990903	Pig	Genotype IX
Uganda2012.Wakiso	Uganda	2012	KC990901	Pig	Genotype IX
Uganda2010.Moyo2	Uganda	2010	KC990898	Pig	Genotype IX
Uganda2010.Adjumani	Uganda	2010	KC990894	Pig	Genotype IX
Uganda2012 Kabale1	Uganda	2012	KC990890	Pig	Genotype IX
KEN 2001.4	Kenya	2001	JX403674	Pig	Genotype IX
UGA2003/1	Uganda	2003	AY351564	Pig	Genotype IX
KEN 2001.1	Kenya	2001	JX403672	Pig	Genotype IX
KEN 2001.5	Kenya	2001	JX403675	Pig	Genotype IX

Uganda2010.Kumi	Uganda	2010	KC990892	Pig	Genotype IX
Uganda2011.Mpigi	Uganda	2011	KC990895	Pig	Genotype IX
Uganda2012.Lira	Uganda	2012	KC990899	Pig	Genotype IX
Uganda2013.Kampala1	Uganda	2013	KC990891	Pig	Genotype IX
Uganda2010.Tororo	Uganda	2010	KC990896	Pig	Genotype IX
UGA/1/95	Uganda	1995	AF449475	Pig	Genotype IX
KEN 2001.3	Kenya	2001	JX403673	Pig	Genotype IX
KEN 2001.6	Kenya	2001	JX403676	Pig	Genotype IX
Uganda2011.Kampala2	Uganda	2011	KC990893	Pig	Genotype IX
Uganda10.Moyo1	Uganda	2010	KC990897	Pig	Genotype IX
Uganda2012.Kampala3	Uganda	2012	KC990900	Pig	Genotype IX
Uganda2010.Amuru	Uganda	2010	KC990902	Pig	Genotype IX
Uganda2012.Kampala4	Uganda	2012	KC990904	Pig	Genotype IX
UGA/3/95	Uganda	1995	AF449476	Pig	Genotype X
ANG/70	Angola	1970	AF301542	Pig	Genotype I
NAM/1/80	Namibia	1980	AF504881	Warthog	Genotype I
ZIM/92/1	Zimbabwe	1992	DQ250119	Pig	Genotype XVII
MAD/1/98	Madagascar	1998	AF270706	Pig	Genotype II
MOZ60-98	Mozambique	1998	AY274455	Pig	Genotype II
NAM/01/95	Namibia	1995	DQ250122	Pig	Genotype XVIII
RSA/1/98	South Africa	1998	AF302818	Pig	Genotype VII
SPEC/154	Botswana	1987	DQ250113	Pig	Genotype V
SPEC/245	South Africa		DQ250117	Pig	Genotype XXII
BOT/1/99	Botswana	1999	AF504886	Pig	Genotype III
RSA/05/95	South Africa	1995	DQ250124	Pig	Genotype III
RSA/01/96	South Africa	1996	DQ250125	Pig	Genotype XXI
MOZ/1960	Mozambique	1960	AF270708	Pig	Genotype V
MOZ/94/8	Mozambique	1994	AF270712	Pig	Genotype VI
RSA/1/95	South Africa	1995	DQ250123	Pig	Genotype XX
RSA/1/99W	South Africa	1999	AF302818	Warthog	Genotype IV
RSA/03/96	South Africa	1996	DQ250127	Pig	Genotype XIX
MOZ/A-98	Mozambique	1998	AY274452	Pig	Genotype VIII
KAB/6/2	Zambia	1983	AY351522	Tick	Genotype XI
MZI/92/1	Malawi	1992	AY351543	Pig	Genotype XII
SUM/14/11	Zambia	1983	AY351542	Tick	Genotype XIII
NYA/1/2	Zambia	1986	AY351555	Tick	Genotype XIV
TAN/2003/1	Tanzania	2003	AY494550	Pig	Genotype XVI
TAN/1/01	Tanzania	2001	AY494552	Pig	Genotype XV

5.3 A copy of the Letter of consent

NATIONAL AGRICULTURAL

RESEARCH ORGANIZATION

NATIONAL LIVESTOCK RESOURCES RESEARCH INSTITUTE
P. O. Box 96Tororo, Uganda
Tel: 045-4448360
045-4437297

INFORMED CONSENT FORM FOR PIG FARMERS

This Informed Consent Form is for pig farmers in areas that reported outbreaks of African swine fever and who we are inviting to participate in research. The title of our research project is "Serological and molecular epidemiology of African swine fever in domestic pigs in Uganda"

Name of Principal Investigator: *Dr. Tonny Kabuuka*

Name of Organization: *National Agricultural Resources Research Institute*

Name of Sponsor: *National Agricultural Research Organization*

PART I: Information Sheet

Introduction

I am Dr. Tonny Kabuuka, working for the National Livestock Resources Research Institute. We are doing research on African swine fever, which is very common in this country. I am going to give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

Purpose of the research

African swine fever is one of the most common and dangerous diseases of pigs in this region. There is currently no vaccine and control is purely supportive treatment and management. The reason we are doing this research is to find out how the causative viruses can be controlled better.

Type of Research Intervention

This research will involve questionnaire administration, blood and tissue sample collection.

Participant selection

We are inviting willing pig farmers in areas that had outbreaks.

Voluntary Participation

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. You may change your mind later and stop participating even if you agreed earlier.

PART II: Certificate of Consent

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research.
Print Name of Participant_____ Signature of Participant _____
Date _____

If illiterate

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness_____ AND Thumb print of participant
Signature of witness _____
Date _____

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1.

2.

3.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF has been provided to the participant.

Print Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____

Day/month/year

5.4 A copy of the questionnaire

SURVEY ON MOLECULAR AND SEROLOGICAL EPIDEMIOLOGY OF AFRICAN SWINE FEVER IN DOMESTIC PIGS IN UGANDA

QUESTIONNAIRE FOR THE PIGGERY FARM HERDS

This questionnaire is being conducted as part of an on-going Master of Veterinary Science study. 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 SECTION A: GENERAL

s/no.						
1	District	2 Sub county				
3	Village					
4	GPS Coordinates	N			E	
5	Name (optional)					
6	Age	**1**<20	**2** 20-30	**3** 31-40	**4** 41-50	**5**> 50
7	Education level	**1** Primary	**2** Secondary		**3** Tertiary	**4** University
8	Main Occupation	**1** Piggery	**2** Crop husbandry		**3** Other animal farming	
9	% time dedicated to pig farming	**1**<1hr	**2** 1-2 hr		**3** 3-4hr	

SECTION B: EPIDEMIOLOGY

10. Types of Pigs	**11. Number**	**12. Age**	**13. Sex**	**14. Source of pig**
1 Local	**1** 1-10	**1** Piglets	1 Male	**1** Market
2 Mixed	**2** 11-50	**2** Growers	2 Female	**2** Neighbouring farms
3 Exotic	**3** 51-100	**3** Adults		**3** Gifts
	4 >100			**4** Other

15. How do you dispose/sell your pig products?	**1** Buyers come to farm	**2** Take to market	**3** Slaughter at home
16. Do farm-gate buyers collect pig/pig product from your farm?		**1** Yes	**2** No
17. Do you have pig abattoir in your premises?		**1** Yes	**2** No
18. Did African swine fever affect your farm in any way?		**1** Yes	**2** No
19. If yes, how (mark as many as applicable)?			
1 Lose pigs	**2** Lose income	**3** Reduction in income	**4** More costs for disease prevention
(This section can be skipped for uninfected farms)			

20. When was your farm infected?	**1** 2009	**2** 2010	**3** 2011	**4** 2012	**5** Other
21. To whom did you report?		**1** Local Vet	**2** DVO	**3** No one	**4** Other
22. How long does it take you between disease infection and reporting?		**1** 24 hr	**2** 1-7 days	**3** 1-2 weeks	**4** 1 month

23. How easily can you report ASF outbreak?			**1** Easy	**2** not easy	**3** Very difficult
24. What did you do after loss of all pigs?		**1**Abandoned piggery	**2** Re-stocked	**3** Tried other stock animals	**4** Other
25. What do you think is responsible for infection in your farm (infected farms only)?					
1 Visiting farmers	**2** Wild pigs	**3** Ticks	**4** Roaming pigs	**5** Own pig roaming	**6** Other
26. How did you sell/dispose of your product during the outbreak?					
1 Rapid slaughter and sale in open market	**2** Destroy and bury/burn	**3** Dispose of in the refuse dump	**4** Slaughter and eat/sell	**5** Government officials handle it	
27. Do you visit other people's farm?				**1** Yes	**2** No
28. Do you have infected farms in immediate neighbourhood?				**1** Yes	**2** No / **3** Not sure
29. How do you dispose your pig intestines and other slaughter waste materials following slaughter procedure?					
1 Burn/bury	**2** Sell for consumption	**3** Dump in refuse site	**4** Dispose indiscriminately	**5** Other (state)	
30. Do wild birds visit your farm?				**1** Yes	**2** No
31. Do these wild birds have access to such intestinal content?				**1** Yes	**2** No
32. Source of feeds	**1** Buy ready-made meal		**2** Compound my animal feed		**3** Leave pig to search
33. Do you see engorged ticks on your pigs?				**1** Yes	**2** No
34. Do you borrow farm equipment?				**1** Yes	**2** No
35. If yes, what?					
36. Source of water				**1 F**armer provides	**2** Not provided
37. Do you share this source with other farms?				**1** Yes	**2** No
38. Any survivor pigs				**1** Yes	**2** No

PART II: BIOSECURITY, MANAGEMENT PRACTICES AND COSTING FOR THE OPERATIONS.

Which of the biosecurity measures tabulated below is practiced or present in the farm? Tick all observed measures.

S/NO	BIOSECURITY MEASURES	Yes	No
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 incoming pigs		
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	Pest control (rodents & insects)		
30	Prompt sick/ dead bird disposal from the farm		
31	Change solution in foot pans regularly		
32	Auditing: incentives, education, adherence (encourage assistants to adhere to biosecurity)		

Thank you for your time