Sveriges lantbruksuniversitet
Fakulteten för Veterinärmedicin och husdjursvetenskap
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Detection of African swine fever virus and phylogenetic characterization of *Ornithodoros* ticks collected from Lake Mburo- and Murchison Falls National Parks in Uganda

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ABSTRACT

This master thesis was performed as a Minor Field Study (MFS), funded by the Swedish International Development Cooperation Agency (Sida), Gulli Strålfeldts fond and the Swedish University of Agricultural Sciences (SLU). The study was performed in collaboration between SLU, the National Veterinary Institute (SVA) in Uppsala and the Makerere University in Kampala, Uganda. The study was part of an on-going project on African swine fever in Uganda called ASFUganda.

African swine fever (ASF) is a devastating haemorrhagic viral disease with a mortality approaching 100% among naïve domestic pigs and currently there is no vaccine available. African swine fever virus (ASFV) is characterized by high stability and effective spread. The virus is transmitted through direct and indirect contact. Pork products stored frozen, salted or smoked may remain infective for months. ASF is endemic in many countries in Africa south of the Sahara where it is circulating in tree different cycles; an ancient sylvatic cycle that involves soft ticks of the genus *Ornithodoros* and wild warthogs, a cycle between *Ornithodoros* ticks and domestic pigs, and a domestic pig cycle that occurs in absence of ticks. Warthogs and soft ticks are considered natural reservoirs of the virus and do not show clinical illness. The epidemiological role of this sylvatic cycle is not fully understood. The aim within this study was to increase the knowledge about the soft ticks role in the epidemiology of ASF. Therefore, in order to clarify how the disease was spread the geographic distribution of different *Ornithodoros* genetic lineage and the prevalence of ASFV in soft ticks collected in two of Uganda's national parks, Lake Mburo and Murchison Falls was investigated and determined. The phylogenetic characterization revealed that the ticks belong to *O. porcinus porcinus* with a small genetic diversity between the two parks. All specimens revealed a close genetic relationship to specimens derived from Tanzania. Virus-DNA was detected in two ticks from two burrows in Murchison Falls NP using a commercial realtime PCR when performed in Uganda. These results were not possible to repeat in Sweden and therefore we were not able to get any sequences for further studies. It is not clear if these two positive samples was a result from contamination or a methodological problem in Sweden.

BACKGROUND

African swine fever virus

History and distribution

Montgomery first described ASF in the 1920s in Kenya. Affected domestic pigs died in a characteristic haemorrhagic fever with a mortality approaching 100%. Pigs that got sick had been in close contact with wildlife suid species particularly warthogs. A virus was identified that did not cause clinical disease among the warthogs. ASF has since its first appearance been described in most sub-Saharan countries (Sánches-Botija, 1982; Wilkinson, 1984).

The first outbreak of ASF outside Africa was reported in 1957 in Portugal. The source of infection was waste containing pork from airplane flights, which was fed to pigs near the airport in Lisbon. The disease was eradicated through testing and slaughter but new outbreaks in Lisbon occurred in the 1960s with further spread to Spain and the virus remained enzootic in the Iberian Peninsula until 1995 when extensive eradication programs were able to fight further spread. *Ornithodoros erraticus* was identified as a vector and reservoir for the virus (Sánches-Botija, 1982; Costard *et al.*, 2009).

Import of infected pork products has been the main source of ASF outbreaks in countries outside Africa. After introduction in an ASF free country, the disease is often transmitted through direct contact between animals and indirect through contaminated vehicles and gears. Transmission also occurs when pigs are fed with infectious and not heat-treated pork products (Sánches-Botija, 1982). Sporadic outbreaks have been reported in many countries including Brazil, Cuba, Haiti, Dominican Republic, Malta, Italy, France, Belgium and the Netherlands. All European countries have been able to eradicate the disease except Sardinia (an Italian island in the Mediterranean) where ASF has remained endemic since its introduction in 1978. The traditional pig production in Sardinia includes free-range pigs with open grazing and small pig holding systems. This system includes contact with wild boars, which make eradication more complicated. In many countries these sporadic outbreaks of ASF have been confused with other swine diseases like Classical swine fever (CSF) and therefore for a time remained unnoticed. This has complicated identification of ASF and there are several examples where an outbreak has been going on for months before a correct diagnosis has been made (Costard *et al.*, 2009; Sánchez-Botija, 1982).

More recent outbreaks outside Africa were reported in 2007-2008 in the Caucasus region (Georgia, Armenia, Azerbaijan) and Russia (Rowlands *et al*, 2007). The first clinical cases in Georgia where seen before May 2007 but were not officially reported to the OIE (World Organisation of Animal Health) until 5 June 2007 (FAO, 2008). Contaminated pork- and pork-products from international ships were most likely the sources for virus introduction. The infection could probably be established since most pigs in Georgia are kept on free range and have access to dumped waste. After introduction the disease spread according to the main

transport routes. The virus isolate belong to genotype II and has close relationship with strains from southeaster Africa (Mozambique, Madagascar and Zambia) (Rowlands *et al*, 2007). The situation in the Caucasus region and Russia is not under control and further spread to eastern Europe is likely (EFSA, 2010). Current distribution of ASFV in the world is demonstrated in Fig 1.

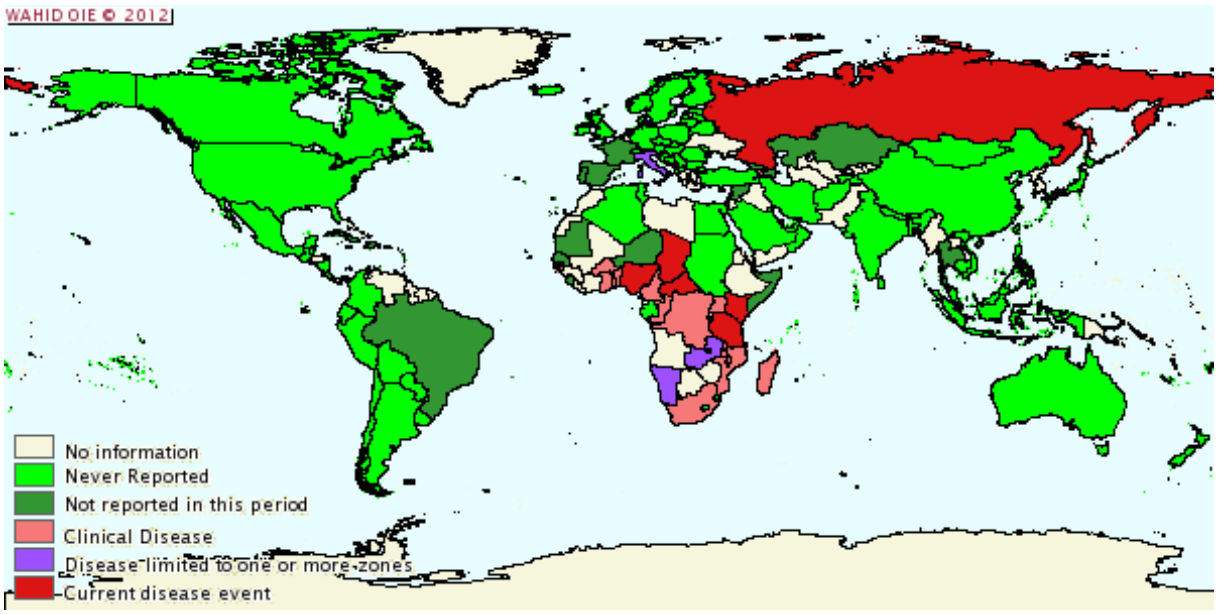

Fig 1. Geographic distribution of ASFV outbreaks (OIE, 2012)

Today ASF is according to OIE one of the most important diseases in domestic pigs. It has major socioeconomic consequences, significant impact on the national trade in pigs and pig products and causes severe animal suffering. ASF is an OIE listed disease and outbreaks must unconditionally be reported (OIE, 2012).

Epidemiology of ASFV

The virus originates from the African continent and the epidemiology of the disease is complex. In many countries south of Sahara the virus circulates in three different cycles: an ancient sylvatic cycle that involves soft ticks of the genus *Ornithodoros* and wild warthogs (*Phacochoerus africanus)*, a cycle between *Ornithodoros* and domestic pigs, and a domestic pig cycle that occurs in absence of ticks (Lubisi *et al.*, 2005).

ASFV can infect all members of the family suidae including domestic pigs, European and American wild boar, warthogs, bushpigs and giant forest hogs. Warthogs, giant forest hogs and bushpigs are together with the tick *Ornithodoros* considered natural reservoirs and do not show clinical illness. Domestic pigs and wild boar on the other hand are susceptible to the virus and the mortality reaches up to 100% among naive animals (Penrith, 2004).

The sylvatic cycle between wild warthogs and the soft tick of the species *Ornithodoros moubata* and *Ornithodoros porcinus* has been present in Africa for a very long time before the first introduction of the disease among domestic pigs in the 1920s. Warthogs are mainly found in eastern and southern Africa and are together with *Ornithodoros spp* considered the major reservoir of ASFV. The *Ornithodoros* ticks, which live in warthog dwellings, get infected with ASFV when feeding on viraemic animals. Only neonate warthogs develop a viraemia with levels high enough ($>10^3$-10^4 HAD_{50}/mL) to be able to transfer the virus to the ticks. The virus maintains its virulence in the ticks and is then able to infect newborn warthogs and the disease is maintained in the sylvatic cycle (Wilkinson, 1984). Adult warthogs on the other hand are considered as dead end hosts because virus concentrations in the blood are generally low ($<10^2$ HAD_{50}/mL) and evidence for vertical or horizontal transmission is absent (Thomson, 1985).

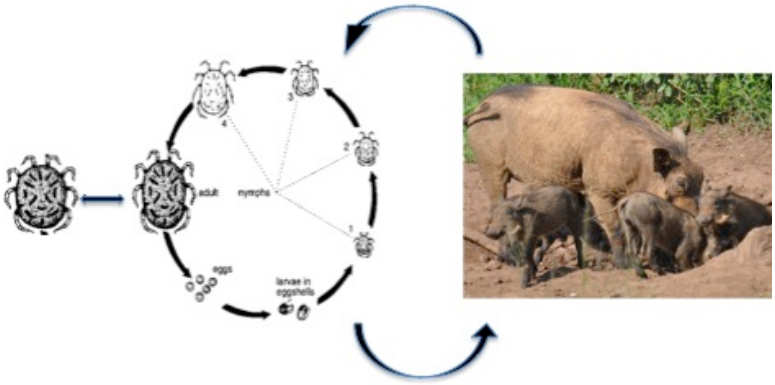

Fig 2. ASFV circulating in the sylvatic cycle between warthog and *Ornithodoros spp*. The virus is spread within the tickpopulation sexually, transstadially and transovarian. *Ornithodoros* get infected when they feed on neonate viraemic warthogs (Modified from Vial, 2009).

Warthogs are mainly grazers and spend the night in burrows in close interaction with *Ornithodoros* ticks (Jori and Bastos, 2009). They are widely distributed in Africa as demonstrated in Fig 3.

In most warthog populations in eastern and southern Africa the seroprevalence of ASFV is high (generally > 80%) but differences in neighbouring populations have been recorded. In some parts of eastern Africa almost 100% of the sampled warthogs had antibodies and at other location, like some parts of South Africa there was almost a complete absence of antibodies in collected samples. An age-related trend has also been reported. In Queen Elizabeth NP in Uganda the sero-prevalence was 58% in 4-12 month old warthogs but decreased to an average of 8% after 24 months of age (Jori and Bastos 2009).

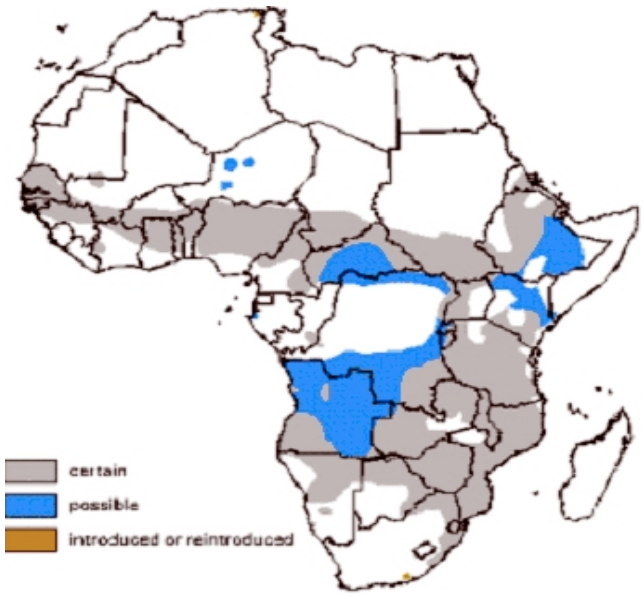

Fig 3. Warthog distribution in Africa (http://www.wild-about-you.com/GameWarthog.htm).

It has never been demonstrated that warthogs can infect domestic pigs through direct contact, but Wilkinson (1984) suggested that domestic pigs might be infected if they eat tissues from warthog carcasses that contain virus. Especially lymphatic tissue can withhold high levels of virus. However, Jori and Bastos (2005) question this route of virus transmission through warthog carcasses since virus levels in lymphatic tissue from warthogs are much lower than those from domestic pigs. They further suggest that it is more likely that pigs become infected after being bitten by ticks, brought to human settlement with warthog carcasses or by ingestion of infected ticks. *Ornithodoros* ticks have occasionally been observed on warthogs outside their burrows and found in intestinal contents of warthog (Jori and Bastos, 2005).

Genetic characteristics of the ASF virus

African swine fever virus (ASFV) is a large DNA virus that used to belong to the family *Iridoviridae* but is now the sole member of the genus *Asfivirus* in the family *Asfarviridae.* It is a complex icosahedral enveloped virus that shows many characteristics similar to *Iridovirus* and *Poxvirus* families (Yáñez, 1995). The genome is double-stranded and varies between 170-190kb. Further, the virus has a conserved central region of 125 kb, the vp72 gene, and variable ends. The high level of diversity of the genome is due to these variable ends, which also determine the virulence in different strains (Yáñez, 1995; Bastos, 2003).

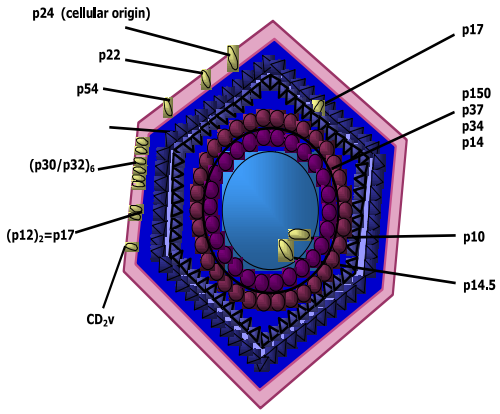

Fig 4. ASFV is a complex icosahedral enveloped DNA virus (EFSA, 2009)

There are 22 genotypes of the virus and molecular phylogenetic analysis shows the presence of 3 distinct evolutionary lineages corresponding to 3 geographically separate locations.

Genotype I, with a highly conserved *vp72* protein (*B646L* gene) has been isolated in 24 countries in Europe, South America, the Caribbean and western and southern Africa. The source of infection is most likely one of the western African countries (Bastos *et al*, 2003). Genotype I has previously been identified in eastern African sylvatic hosts such as bushpigs and ticks, which according to Lubisi *et al* (2005) is notable since it only occurs among domestic pigs in western Africa. In the same study Genotype V, X and XII was found in both domestic pigs, wild

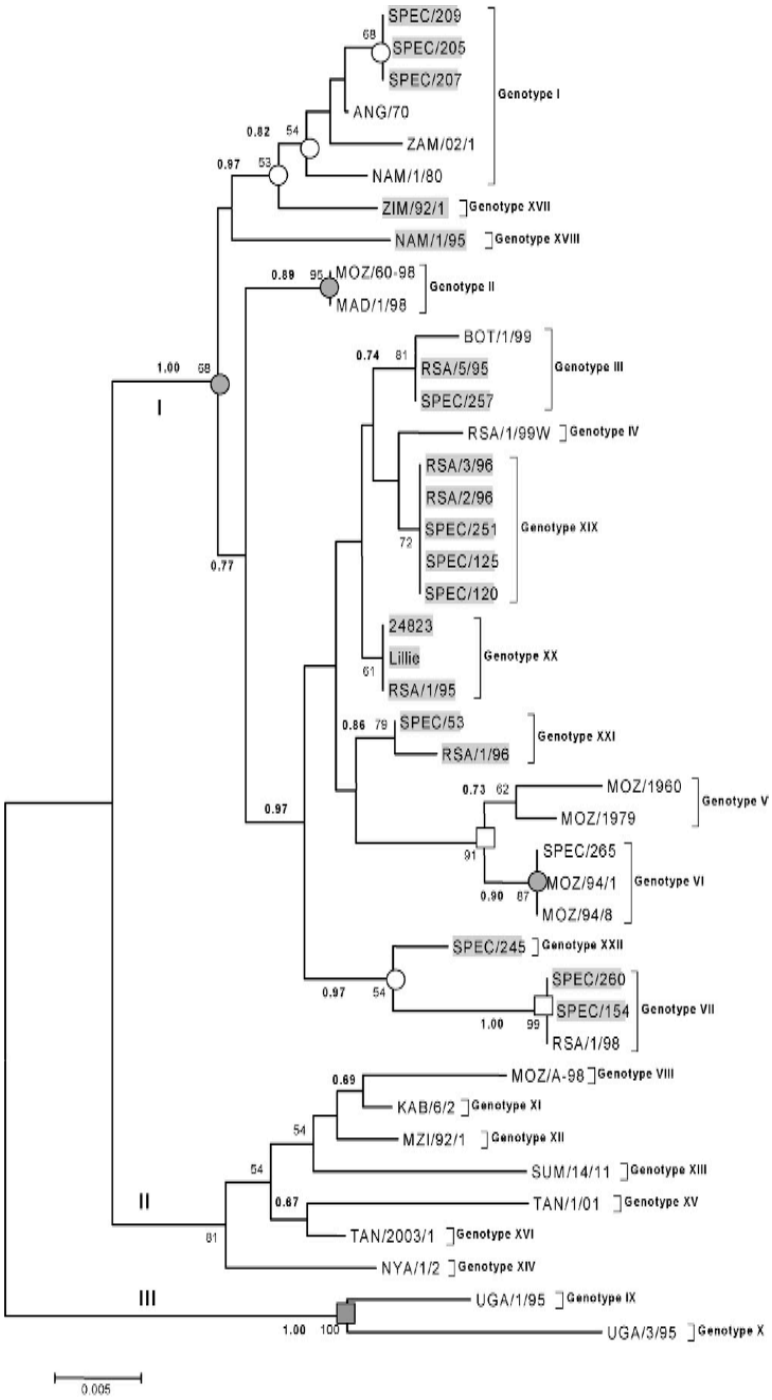

Fig 5. Neighbor-joining tree describing 3 distinct evolutionary lineages corresponding to 3 geographically separate locations

suids and ticks, indicating that it occurs as a spill-over from the sylvatic cycle to domestic pigs. Genotype XI, XIII and XIV appears to be completely associated with the sylvatic cycle since they all originate from ticks in warthog burrows. Genotype II, VI, VII, IX and XVI on the other hand was only found among domestic pigs (Lubisi *et al*, 2005). Genotype IX is found among domestic pigs in Uganda, Congo (Gallardo, 2011) and Kenya (Gallardo, 2008). Genotype X has been isolated from domestic pig, ticks and warthogs and has been found in Uganda, Burundi, Tanzania and Kenya (Lubisi *et al*, 2005; Boshoff *et al*, 2007).

Pathogenesis

Variation in virulence combined with host-factors provides several disease forms, ranging from highly lethal to subclinical forms. If the susceptible animals are infected with highly virulent strains the result is severe haemostatic and hemodynamic damage with a devastating outcome (Kleiboeker, 2001; Rock, 2001).

Cells in the mononuclear phagocyte system are primarily infected; these include tissue macrophages and specific linear of reticular cells (Yáñez, 1995). Macrophages are responsible for antigen-presentation and development of acquired immune responses. Therefore a dysfunction in these cells has a severe effect on the host immune response. The ability to infect and replicate in macrophages appears to be the critical factor in ASFV virulence. Moderately virulent strains do also infect this type of cells but the damage is much less severe (Yáñez, 1995). In the infected macrophages ASFV inhibits expression of proinflammatory cytokines such as tumour necrosis factor alpha (TNFα) and interferon alpha. There are also two genes that modulate cellular apoptosis in infected macrophages but also in uninfected T and B lymphocyte population and megacaryocytes (Powell, 1996). Increased TNF α expression has also been reported during infection with ASFV and this may play a key role in the pathogenesis concerning changes in vascular permeability, coagulation, and induction of apoptosis in uninfected lymphocytes (Gómez del Moral, 1999).

Stability of the virus

The virus is highly resistant and can survive freezing, moderate heat (56°C/70 minutes; 60°C/20 minutes) and extreme pH values. Its resistance is increased if the virus is present in serum. Pork can stay infectious for months if the meat is frozen, salted or smoked (EFSA, 2009).

Fig 6. ASFV is highly resistant and infected pork products can stay infectious for months if the meet is stored frozen, salted or smoked. Picture taken in Gulu district, Uganda, Dr Tony Aliro, District Veterinary Officer, Gulu.

Clinical symptoms in domestic pigs

The clinical forms of African swine fever are divided into four groups, peracute, acute, subacute and chronic. In natural infection with ASFV, clinical signs appear approximately 5-15 days after infection. Clinical African swine fever cannot be separated from Classical swine fever based on clinical signs. To establish which virus that is causing the disease laboratory methods are required (FAO, 2000).

Peracute/acute

The mortality in peracute and acute form is almost 100%. Pigs dying in the peracute form are affected with strains of high virulence and they die without showing clinical symptoms, or possibly a short period of high fever (Epiwebb, 2010). High fever is also characteristic in the acute form. The body temperature reaches over 40 ^{o}C, the pigs get depressed and lose their appetite. Respiratory problems and vomiting are common and mucopurolent discharges from nose and eyes are sometimes seen. The hind legs appear week and many animals show an unsteady gait. Some animals develop bloody diarrhoea while some become constipated and symptoms of abdominal pain can appear. Bleeding from mouth and rectum can also be observed in some of the animals. Mucous membranes are red and congested and the skin can turn cyanotic especially on the ears, nose, abdomen, gluteus and the lower extremities. Abortion is common among pregnant sows. After developing clinical signs pigs usually die within one to seven days (FAO, 2000; epiwebb, 2010). Before death they become comatose due to fluid in the lungs or as a result of developing haemorrhagic shock (FAO, 2000).

Subacute

Domestic pigs get infected with moderate virulent strains in the subacute form. This is most common in areas where the disease is enzootic. The mortality depends on age, nutrition status and general condition among the animals. The development of disease is not as rapid as in the acute form but last a period of 2-3 weeks and the symptoms are characterized by a constant high or fluctuating fever. In addition to the fever they show symptoms like the acute form but milder. Surviving animals can recover or become chronic infected (FAO, 2000).

Chronic

The chronic form is seen in Portugal and Spain and is associated with the use of poorly attenuated vaccine strains. Symptoms vary but include, stunted growth, emaciation, dermatitis and arthritis (FAO, 2000).

Ornithodoros - soft tick

Taxonomy

The genus *Ornithodoros* belongs to the family *Argasidae*, a complex and diverse collection of about 190 species (Estrada-Peña *et al.*, 2010). *Ornithodoros* consist of approximately 90 species of which 10 are of veterinary importance (Taylor *et al.,* 2007). The lack of adequate guidelines based on morphological characteristics and the high biodiversity of *Argasidae* has made the taxonomy complicated. As a consequence, the taxonomy among *Ornithodoros spp* in the literature is indeterminate and confusing (Estrada-Peña *et al.*, 2010). The species that are considered reservoirs for ASFV in the African sylvatic cycle are referred in the literature as *Ornithodoros moubata*, *Ornithodoros moubata porcinus*, *Ornithodoros porcinus* or *Ornithodoros porcinus porcinus* (Kleiboeker and Scoles, 2001).

Ornithodoros moubata was identified by Walton in 1962. He suggested in 1979 that it was a species complex which could be divided into at least four different species *O. compacticus, O. apertus, O. porcinus porcinus* (associated with warthog burrows) and *O. porcinus domesticus* (found in human dwellings). However Bastos *et al* published in 2009 a phylogenetic study, which did not support the division into two separate subspecies within O. *porcinus*. Instead, three geographically distinct *O. porcinus* lineages were revealed. O. *moubata* appears to be related to one of the three geographically separated O. *porcinus* lineages (Fig. 7).

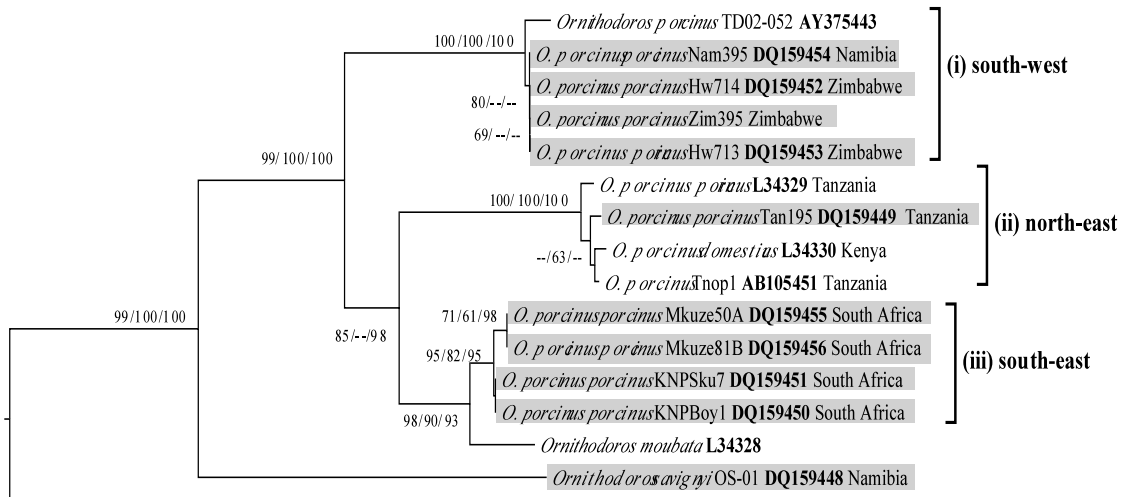

Fig 7. Phylogenetic tree of *Ornithodoros porcinus porcinus* showing three geographically distinct lineages (Bastos *et al.*, 2009).

Morphology

Argasidae lack a scutum, the hard plate on the dorsal surface that Ixodid (hard tick) have. Larvae have three pairs of legs, while nymphs and adults have four pairs. Adults have a large genital aperture that the nymphal stages lack. Male and females are not possible to distinguish (Estrada-Peña *et al.*, 2004).

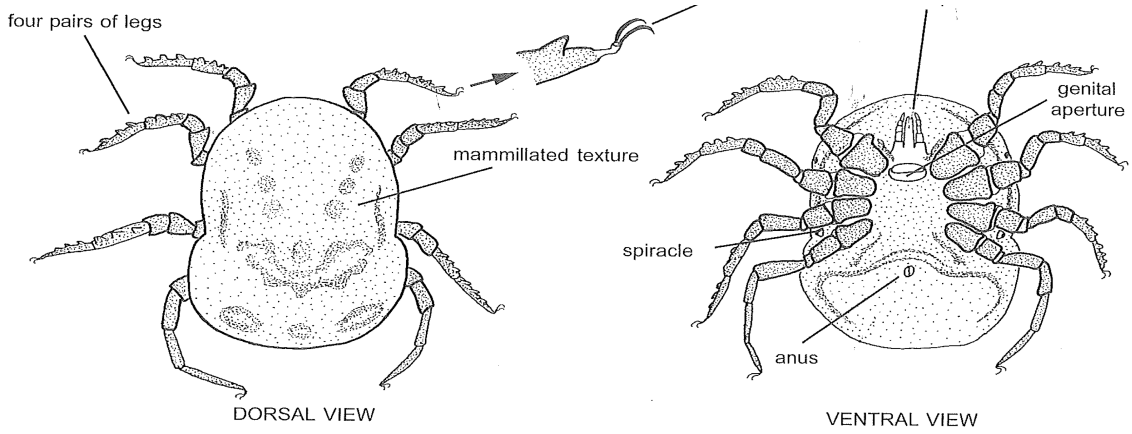

Fig 8. Morphology of *Ornithodoros* ticks. The genital aperture distinguish adults from nymphs (Estrada-Peña *et al.*, 2004).

Distribution of Ornithodoros spp in the world

Ornithodoros spp are common in tropical and subtropical areas (Vial, 2009). Most species are found in Africa but they do also occur in North America, South America, Asia and Europe (Taylor *et al.,* 2007).

The 10 *Ornithodoros spp* that are considered of veterinary importance include *O. erraticus, O. hernsi, O. moubata, O. porcinus porcinus, O. parkeri, O. savignyi, O. tholozani, O. turicata, O. rudis, and O. lahorensis* and their geographic distribution, host preference and pathogen is shown in table 1 (Taylor *et al.,* 2007).

Species	Host	Geographic distribution	Pathogen
O. erraticus	Mainly small animal but also domestic livestock and human	Europe, Africa, Middle east	ASF, Q fever, Babesia,Tick borne relapsing fever (TBRF),
O. hernsi	Most mammals	North America	ASF, TBRF
O. moubata	Most mammals, birds and some reptiles	Africa and the Middle East	ASF African relapsing fever, Q fever
O.porcinus porcinus	Warthog, bushpig, porcupines, domestic pigs	Africa, Madagascar, southern Europe	ASF,
O. parkeri	Most mammals particularly rodents	Western states and Pacific coast of North America	ASF, TBRF,
O. savignyi	Most mammals, particularly camels and poultry	Africa, India, the Middle east	Toxicosis
O. tholozani	Most mammals, birds and reptiles	Africa, the Middle east	Persian and relapsing fever
O. turicata	Most mammals, particularly rodents	North America	Q-fever, TBRF
O. rudis	Most mammals, particular rodents and humans	Central and South America	TBRF
O. lahorenis	Wild sheep, domestic sheep and goats	Eastern Europe, Northern India, Middle east	Piroplasmosis, brucellocis, Q-fever, tularaemia

Table 1. *Ornithodoros spp* geographic distribution, host preference and pathogens that have been associated to each species (Modified from Taylor *et al.,* 2007).

Habitats

Ornithodoros spp live in dwellings of vertebrate animals like nests, caves and burrows. Ticks may also hide in cracks in human and livestock dwellings or in rocky, sandy or dusty ground. The ticks are able to survive at very simple environmental conditions, and are tolerant to high temperatures and low humidity. Even if soft ticks are able to survive under harsh conditions they depend more on optimal surroundings to develop. This is established by shelter in microhabitats combined with their host's body heat and moisture from the respiration and excrement (Vial, 2009).

Fig 9. Picture to the left showing warthog burrows in Murchison Falls NP. The picture to the right is taken in Gulu district and is an example on a pig holding system where ticks can hide in cracks and ground surface.

Lifecycle

Ornithodoros ticks do not stay attached to their host since they have a rapid feeding process. Particularly nymph and adult feed rapidly, 15-60 min in average. The development stages correspond to other mites, i.e. larvae, nymphs and adults. Depending on species the amount of nymph moults varies between two to eight and each stage requires at least one blood meal. *Ornithodoros moubata* and O. *porcinus* have several instars, which requires 76-155 days to develop. The adult female lays batches of eggs (5-500) after each blood meal and she is able to produce up to five clutches during her lifetime. *Ornithodoros* ticks are able to survive up to 25 years if they have access to regular feeding and large nymphs and adults can survive periods of starvation for several years (Vial, 2009). Since soft ticks are relatively fast feeding it is difficult to investigate their host specificity but in general the burrow-inhabiting *Ornithodoros spp* seem to have no or little host specificity and feed on any hosts available (Taylor *et al.*, 2007).

Fig 10. Adult *Ornithodoros* tick from a warthog burrow in Uganda. Picture taken by Karl Ståhl.

Pathogenesis of ASFV in Ornithodoros ticks

Experimental infection with ASFV and transmission to pigs has been performed with several species, which indicate that all ticks from genus *Ornithodoros* may be potential vectors of the virus (Kleiboeker *et al.*, 1998).

It is mainly adults and older nymphs that are able to feed enough amount of blood to get infected with virus and this is probably the reason why the rate of infected ticks increases with larger and older ticks. The prevalence of infected ticks may be as high as 10% in a burrow hosting ASFV (Wilkinson, 1984). The virus is well adapted to its vector and persists there probably for life (Wilkinson, 1984; Kleiboeker and Scoles, 2001). Kleiboeker *et al* showed in 1998 that in *O. moubata* the virus first replicates in the midgut epithelium and then undergoes secondary replication in the salivary and coxal glands, hemocytes and reproductive and connective tissues. Furthermore the virus remains conserved in all tissues. The virus persists its virulence through all developing-stages and since the reproductive organ is infected the virus can spread in the population sexually during mating and transovarially to the offspring (Kleiboeker *et al.*, 1998). Transovarial transmission of ASFV varies between ticks and batches of eggs from the same tick. Increased number of blood meals increases the rate of laying batches of infected eggs (Rennie *et al.,* 2001). Transstadial transmission in O. *erraticus* – the species found in Europe was observed by Sanchez-Vizcaino (2006). All species of genus *Ornithodoros* are considered potential reservoir of ASFV.

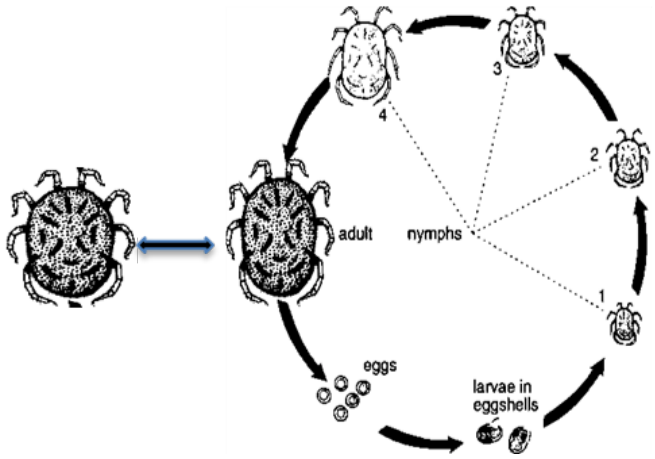

Fig 11. The virus persists its virulence through all developing-stages and since the reproductive organ is infected the virus can also spread in the population sexually during mating and transovarially to the offspring (Modified from Vial, 2009).

Transmission to pigs was shown by Kleiboeker *et al* (1998) to have two potential routs. Due to virus in the salivary gland, ticks are able to transfer virus to pigs when feeding. A second possibly rout for transmission is through coxial fluid which the tick secret during or shortly after feeding. Virus is detected in the coxial fluid from the majority of infected ticks and in

the study by Kleiboeker *et al* (1998) 100 % of the coxial fluid contained virus but only 50 % of the same ticks had virus in the salivary secretions.

INTRODUCTION TO THE STUDY

In Uganda, one of the countries where ASF is endemic, two ASFV genotypes has been described, genotype IX and X. Genotype IX is circulating among domestic pigs in Uganda and has been reported as cause of outbreaks since 1995. This genotype has also been found in Kenya (Gallardo *et al.*, 2008) and recently in Democratic Republic of Congo (Gallardo *et al.,* 2011). Genotype X has been found in the neighbouring countries Burundi, Tanzania and Kenya were it has been isolated from domestic pigs, ticks and warthogs (Lubisi *et al.*, 2005; Boshoff *et al.*, 2007). In Uganda genotype X has only been detected in ticks were it has been isolated from one of Uganda's National Park, Lake Mburo (unpublished report, Ståhl and others, 2011).

Clarifying the genetic distribution of different *Ornithodoros* genetic lineages and prevalence of ASFV is a way to increase the knowledge of soft ticks role in the epidemiology of ASF. There are no previous studies on the taxonomy of *Ornithodoros spp* described from Uganda and since it seems to be a different genotype that circulates in the sylvatic cycle it is also interesting to investigate not only the prevalence but also to sequence the virus to se what genotype that circulates among the population in Uganda.

Aim of the study

The aim with this study was to increase the understanding of *Ornithodoros* ticks role in the epidemiology of ASF. To achieve these objectives this study consisted of two parts:

1. Fieldwork that involved collecting soft ticks in two of Uganda's National Parks, Lake Mburo and Murchison Fall.
2. Laboratory work performed at Makerere University, Kampala, Uganda and at SVA, Uppsala, Sweden. The laboratory work included:
    a. Total genomic extraction from *Ornithodoros* ticks
    b. PCR amplification, and sequencing of selected sections of the tick genome
    c. Phylogenetic characterization of *Ornithodoros* ticks derived from the two National Parks followed by a comparison of the diversity between ticks from different burrows located in the same national park and between the parks
    d. Determine presence of ASFV in collected ticks and sequence the virus

MATERIAL AND METHODS

Tick collection, sorting and storage

Ticks from a total of 11 burrows at different locations in Murchison Falls National Park and 11 burrows from Lake Mburo National Park where collected. A long shovel was used to pick soil that was manually removed and placed in sunlight on a clean plastic sheet. Since *Ornithodoros* ticks are photophobic they start to move and could easily be seen and collected. All ticks were put in glass jars with some soil and holes for breathing in the lid.

Fig 12. Map of Uganda and the National Parks Lake Mburo and Murchison Falls (http://www.tours2eastafrica.com/t2ea_uganda.html)

Fig 13. Fieldwork in Lake Mburo and Murchison Falls NP. A long shovel was used to pick soil that was manually removed and placed in sunlight. Since *Ornithodoros* ticks are photophobic they start to move and could easily be seen, collected and put in glass

Back at the lab at Makerere University in Kampala, the ticks were first killed through freezing at -80 ^{o}C and then sorted. Since the nymphs vary much in size, they were divided into three groups according to size: nymph 1 was the largest and nymph 3 the smallest. No larvae were found. Adults were separated from nymphs by identifying the genital aperture (Estrada-Peña *et al.*, 2004). The sorted ticks were stored in -80 ^{o}C until further investigation. The results are shown in table 2 and 3.

Table 2. Ticks collected in Murchison Falls national park

Park	Burrow ID	*Adult*	*Nymph 1*	*Nymph 2*	*Nymph 3*	**Total**
MF	mf2	1	4	15	7	27
MF	mf3		2			2
MF	mf4	9	20	13		18
MF	mf6	5	3	2		10
MF	mf7	1	1	1		3
MF	mf8	15	23	9	1	48
MF	mf14	5	17	3		25
MF	mf15	5	4			9
MF	mf17	18	33			51
MF	mf18	1	2			3
MF	mf19	12	29			41
Total						266

Table 3. Ticks collected in Lake Mburo national park

Park	Burrow ID	*Adult*	*Nymph 1*	*Nymph 2*	*Nymph 3*	**Total**
LM	lm1	5	9	10	13	42
LM	lm3		1	1		2
LM	lm4	1	5	5		11
LM	lm5		1			1
LM	lm6	2		1		3
LM	lm7		1	2		3
LM	lm8		13	25	3	41
LM	lm9	3	6	1	1	10
LM	lm10	5	8	2		15
LM	lm11		2			5
LM	lm12	1	7	20	30	58
Total						191

DNA extraction and storage

Extraction of nucleic acids from the ticks was performed with a method modified after Ravaomanana (2010) using DNeasy Blood & Tissue kit, QIAGEN, Hilden, Germany. 138 ticks were selected from different developmental stages and from each one of the burrows in the two parks. Each tick was washed twice with sterile water and cut with a scalpel. 200µl phosphate-buffered saline (PBS) was added and the tick was then crushed with sterile sand using a thin plastic pestle. 180ul 98% ethanol and 10 µl proteinase K was added and incubation occurred at 56^{o}C overnight. Extraction was then performed and the total amount of extracted material was 300µl. The extracted material was stored in 1.5 ml Eppendorf tubes at -20 ^{o}C. The total amount of extracted ticks is shown in table 4.

Table 4. DNA-extraction

Park	*Adults*	*Nymph 1*	*Nymph 2*	*Nymph 3*	*Eggs*	**Total**
Murchison Fall	20	34	15	2	2	73
Lake Mburo	13	36	16			65
Total	33	70	31	2	2	138

Tick-DNA amplification and sequencing

To determine the phylogenetic relationship between ticks collected from different burrows samples were selected from each of the 11 burrows from the two national parks. The *16SrDNA* of each tick was amplified by polymerase chain reaction (PCR) according to a protocol designed by Bastos *et al*. (2009). The invertebrate host primers, 16SFArg 5'-GGACAAGAAGACCCTATGAAT-3' and 16S-RArg 5'-CCGGTCTGAACTCAGATCA-3' that were selected are designed to target a completely conserved region within the aligned mitochondrial *16S rRNA* gene of 10 Argasid sequences. This vide spectrum should therefore include all *Ornithodoros spp*.

The amplified product was then further evaluated on gel electrophoresis using agarose gel (40 mM Tris–acetate, 2 mM EDTA, pH 8.3) stained with ethidium bromide. The extracted material was also evaluated on a general DNA level to make sure that DNA was present in the extracted material.

The bands of the correct size were excised and tick DNA was purified from the gel using Qiaquick Gel Extraction Kit, Qiagen. The amplified and purified DNA-products were sent for sequencing.

Detection of African swine fever virus

The extracted material from the 138 ticks were analysed for ASFV using two different PCR protocols. A commercial realtime PCR - ASFV PCR Assay, Tetracore, Rockville, USA (run on SmartCycler, Cepheid Inc., Sunnyvale, California) was used for the first screening. Then as ticks are known to contain inhibitory substances for the PCR reaction a nested protocol was also used (Bastos, 2006). 70 samples were selected and evaluated using Nested PCR that amplified part of the vp72 gene described by Bastos (2006). The primers used amplified fragments of 370 bp in the first round and 243 bp in the second round. Thermal cycling conditions were: 95◦C for 7min; 35 cycles of 95◦C for 30sec., 60◦C for first-round PCR or 61◦C for nested PCR for 30 sec., 72 ◦C for 30 sec.; and a final elongation at 72 ◦C for 10 min.

The amplified products were then further evaluated on gel electrophoresis using 1,5% agarose gel (40 mM Trisacetate, 2 mM EDTA, pH 8.3) stained with ethidium bromide. Bands of correct size were cut out for purification and sequencing.

RESULTS

Phylogenetic characterization

DNA was present in extracted samples when evaluated by a general DNA concentration measure, but when the *16SrDNA* fragment was amplified it was only possible to get products from 14 samples. The samples came from 7 different burrows, 4 samples from Lake Mburo NP from 3 different burrows and 10 samples from Murchison Falls NP from 4 different burrows. When sequenced, the result show that ticks from Lake Mburo and Murchison Falls separates into two different lineages corresponding to which park they origin from (fig 14). Based on this very conserved part of the genome it was not possibly to differentiate between ticks from different burrows in the same park. The phylogenetic relationship was evaluated as the sequences where compared with sequences available in GenBank which revield that they cluster together with *O. porcinus porcinus*. The sequences from Lake Mburo (LM) match well with L34329.1 and the ones from Murchison (MF) with DQ159449.1. These sequences were genetically similar to ticks derived from Tanzania described in Bastos *et al.* (2009).

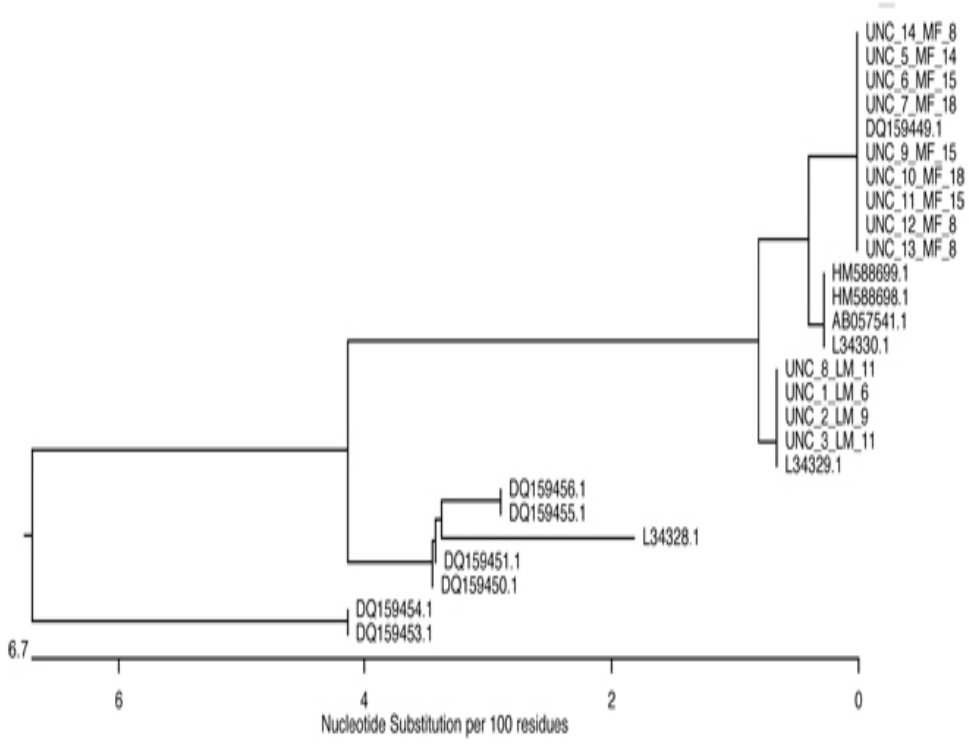

Fig 14. Phylogenetic tree of ticks collected in Lake Mburo (LM) and Murchison Fall (MF) NP. The phylogenetic characterisation shows that the ticks belong to *O. porcinus porcinus* with a small genetic diversity between the two parks. No diversity within the same park was present.

ASFV DNA detection

Two positive samples from two burrows (6 and 8) in Murchison Fall NP were detected using the commercial realtime PCR (fig 15) when the analysis was performed in the Molecular biology laboratory at Makerere University in Kampala, Uganda. The two positive samples were then further tested using the same techniques at SVA in Uppsala, Sweden, with negative outcome. The remaining samples tested negative for ASFV.

From the nested PCR 5 of the 70 samples had bands in the same DNAsize as the positive control when evaluated on gel electrophoresis. No virus was however detected when the samples were sequenced.

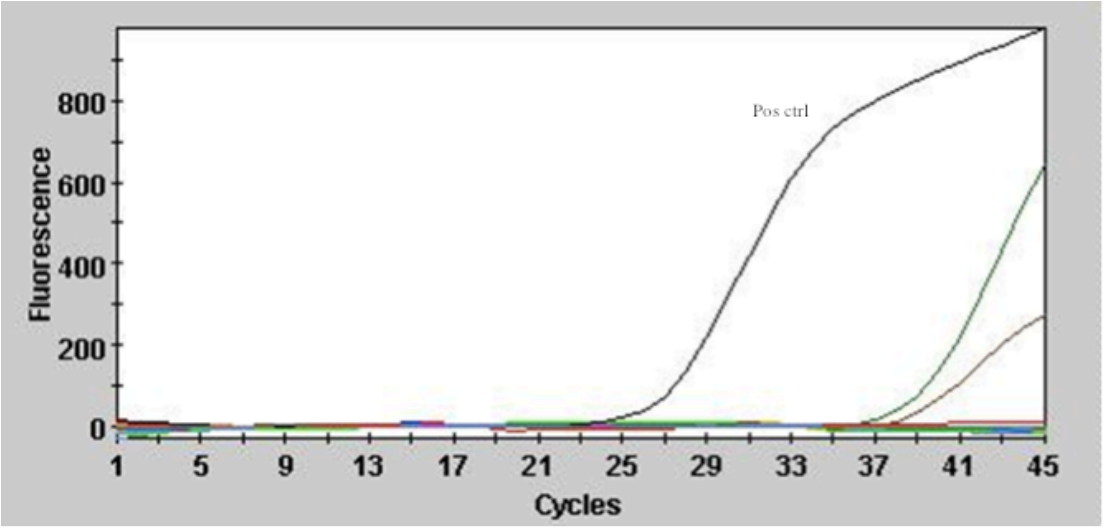

Fig 15. Two positive ASFV samples from burrow 6 and 8 from Murchison Fall NP. Detected with commercial Realtime PCR in Uganda. The result could not be repeated in Sweden.

DISCUSSION

Clarifying the genetic distribution of different *Ornithodoros* genetic lineages and prevalence of ASFV is a way to increase the knowledge of the soft ticks role in the epidemiology of ASF. In Uganda the virus that circulate in the domestic pig population belong to genotype IX and the one found among ticks belong to genotype X. This makes it interesting to investigate not only the prevalence of ASFV among the ticks but also to sequence the virus and determinate the genotype to se if it occurs a spill over from the sylvatic cycle.

The phylogenetic analysis of *Ornithodoros* ticks from Lake Mburo and Murchison Falls NP revealed a close genetic relationship between these and ticks derived from Tanzania, both belonging to *O. porcinus porcinus* one of the natural reservoirs of ASFV. Furthermore it does also appear to be some small diversity among ticks from the two parks since the sequences show some differences. Within the same park no diversity was however detected. *O. porcinus*

porcinus is considered as the natural reservoir of ASFV and this study support the fact that ASFV is circulation in an sylvatic cycle in Uganda.

Analysing ticks is complicated since the tissue contains PCR inhibiting factors, which are preserved in the extracted material and as a consequence of this providing an increased risk of false negative results. For example, it was not possible to amplify tickDNA from each burrow in the two parks. Furthermore these PCR-inhibiting factors derived from the tick could be the reason why the two samples from Murchison Fall NP first were detected as positive using realtime PCR in Uganda but were negative when the analyse was repeated in Sweden using the same technique. The positive samples could of course also be the result from contamination in the lab in Uganda even if strict rules of good laboratory practice were used to avoid this.

Techniques based on sensitive realtime PCR provide a rapid method of diagnosing ASFV in clinical samples from live pigs. However this technique is maybe not as reliable when handling tissue-samples from ticks. Since ASFV has very effective transmission-routes in *Ornithodoros* ticks (sexually, transstadially and transovarian) the prevalence of ASFV in virus-positive burrows should theoretically be much higher than the around 10% found by Wilkinson (1984) in positive burrows. This low prevalence could be a consequence of the methodological problems in detecting virus. The two-step nested PCR developed by Bastos (2006) provides a more sensitive analyse technique but is more time-consuming and the risk for carry-over contamination is increasing. According to Bastos (2006) the nested PCR is the most reliable test analysing ASFV but in this study it was not possible to get any sequence data using this technique. A reason for this might be that the protocol has to be modified and the sample material diluted so the amount PCR-inhibitors decreases.

Ornithodoros ticks are fascinating as survivors since they are able to feed on different hosts, are long living and have potential to survive starvation for a remarkable long time. This knowledge together with the fact that ASFV persist in the tick and have effective transmission to both ticks and pigs indicates that establishing infected tick population within domestic pig facilities would be devastating for future pig production in the affected building. This fact does also complicate eradication of ASFV in endemic areas since the ticks most likely always will be present. Increased knowledge of *Ornithodoros* ticks role in the epidemiology of ASFV is important and requires more studies.

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