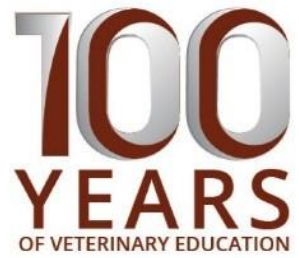




UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Faculty of Veterinary Science

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Interrelationships of warthogs (*Phacochoerus africanus*), *Ornithodoros* ticks and African swine fever virus in South Africa

Anthony Francis Craig



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Submitted in partial fulfilment of the requirements for the degree
Doctor of Philosophy (PhD) in Veterinary Science

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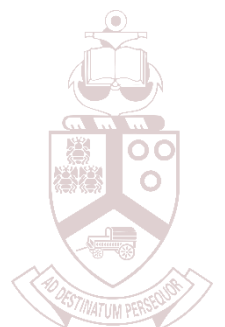
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PREFACE

This thesis consists of a review of relevant literature plus motivation, three published manuscripts and a concluding chapter which briefly summarises the findings and makes recommendations for future research.

DECLARATION

I declare that the thesis, which I hereby submit for the degree Doctor of Philosophy (PhD) in Veterinary Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

ETHICS STATEMENT

The author, whose name appears on the title page of this thesis, has obtained, for the research described in this work, the applicable research ethics approval and declares that he has observed the ethical standards required in terms of the University of Pretoria's Code of Ethics for Researchers and the Policy guidelines for responsible research.

DISCLAIMER

This thesis includes three chapters prepared as stand-alone papers that have already been published in separate scientific journals. Consequently, unavoidable overlaps and repetitions occur between chapters, and the reference style and format differ between chapters.

A handwritten signature in black ink, appearing to be 'AF Craig', with a large loop at the start and a trailing flourish.

Mr AF Craig

PhD Candidate

CONTRIBUTIONS OF THE AUTHORS OF THIS THESIS

The following researchers contributed to the thesis:

Mr AF Craig – Responsible for compiling the background information and motivation, literature review, design and planning of the research manuscripts, analyses and interpretation of results and inscription of all sections forming this thesis.

Prof R Swanepoel – Supervisor of the thesis and principal investigator of the study. Responsible for intellectual and technical input, evaluation of analyses, design and planning the research manuscripts and thesis.

Prof JE Crafford – Co-supervisor of the thesis. Responsible for intellectual and technical input, evaluation of analyses, design and planning the research manuscripts and thesis.

The following statement from the researchers confirms their individual involvement in this study and gives their permission that the relevant research manuscript(s) may form part of this thesis.

Hereby, I declare that I approved the abovementioned thesis and that my role in this study (as stated above) is representative of my contribution towards the research manuscript(s) and supervised PhD study.



Prof R Swanepoel

Supervisor



Prof JE Crafford

Co-supervisor

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LIST OF PUBLICATIONS AND CONFERENCES

Publications

Craig AF, Heath L, Crafford JE, Richt JA and Swanepoel R (2021) Updated distribution and host records for the argasid tick *Ornithodoros (Pavlovskyella) zumpti*: a potential vector of African swine fever virus in South Africa. *Onderstepoort Journal of Veterinary Research*.

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Craig AF, Schade-Weskott ML, Harris HJ, Heath L, Kriel GJP, de Klerk-Lorist L-M, van Schalkwyk L, Buss P, Trujillo JD, Crafford JE, Richt JA and Swanepoel R (2021) Extension of sylvatic circulation of African swine fever virus in extralimital warthogs in South Africa. *Frontiers in Veterinary Science*.

<https://www.frontiersin.org/articles/10.3389/fvets.2021.746129/full>

Craig AF, Schade-Weskott ML, Rametse T, Heath L, Kriel GJP, de Klerk-Lorist L-M, van Schalkwyk L, Buss P, Trujillo JD, Crafford JE, Richt JA and Swanepoel R (2022) Detection of African swine fever virus in *Ornithodoros* tick species associated with indigenous and extralimital warthog populations in South Africa. *Viruses*.

<https://doi.org/10.3390/v14081617>

Conferences

A.F. Craig, L. Heath, J.E. Crafford, and R. Swanepoel – Interrelationships of warthogs, *Ornithodoros* ticks and African swine fever virus in South Africa. Southern African Society for Veterinary Epidemiology & Preventive Medicine 18th Annual Congress 2021.

A.F. Craig, M.L. Schade-Weskott, H.J. Harris, L. Heath, D. Kriel, L de Klerk-Lorist, L. van Schalkwyk, P. Buss, J.D. Trujillo, J.E. Crafford, J.A. Richt and R. Swanepoel – Extension of sylvatic circulation of African swine fever virus in extralimital warthogs in South Africa. University of Pretoria, Faculty of Veterinary Science, Annual Veterinary Faculty Day 2021.

LIST OF ABBREVIATIONS

%	Percentage
µL	microlitre
°C	Degrees Celsius
AbALV	Abalone asfa-like virus
Addo ENP	Addo Elephant National Park
AgriSETA	South African Agricultural Sector Education and Training Authority
ARC-OVR	Agricultural Research Council-Onderstepoort Veterinary Research
ASF	African swine fever
ASFV	African swine fever virus
AVKR	Andries Vosloo Kudu Reserve
bp	Base pairs
BSL	Biosafety Laboratory
COVID-19	Coronavirus disease 2019
Cq	Quantification cycle
CVR	Central variable region
DALRRD	Department of Agriculture, Land Reform and Rural Development
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of the Congo
ECP	Eastern Cape Province
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
FSP	Free State Province
GKNP	Greater Kruger National Park
GP	Gauteng Province
HAD	Hemadsorption
hpi	Hours post infection
IgG	Immunoglobulin G
Kb/kbp	Kilo-base pair
KZNP	KwaZulu-Natal Province
Km	Kilometers
LP	Limpopo Province
Mokala NP	Mokala National Park
MP	Mpumalanga Province
NCP	Northern Cape Province
NCS	Nobuto cellulose strips

NGS	Next-generation sequencing
Nt	Nucleotide
NTC	No template control
NWP	North West Province
OIE	Office International des Epizooties: World Organisation for Animal Health
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PHASA	Professional Hunters Association of South Africa
QGIS	Geographic Information System
QDGC	Quarter-degree grid cell
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
Rolfontein NR	Rolfontein Nature Reserve
SANParks	South African National Parks
SNP	Single nucleotide polymorphism
TADL	Transboundary Animal Diseases Laboratory
TBRF	Tick-borne relapsing fever
UP	University of Pretoria
VP	Viral protein
w/v	Weight Volume
WOAH	World Organization for Animal Health
WCP	Western Cape Province

SUMMARY

Sylvatic circulation of African swine fever virus (ASFV) in warthogs and eyeless *Ornithodoros* ticks that live in warthog burrows historically occurred in northern South Africa. Outbreaks of the disease in domestic pigs originated in this region. A controlled area was declared in the north in 1935 and regulations were implemented to prevent transfer of potentially infected suids or products to the rest of the country. However, over the past six decades, warthogs were widely translocated to the south where the extralimital animals flourished to become an invasive species. Initial transfers were made from a source considered to be free of ticks and virus, but multiple unrecorded translocations accompanied the subsequent burgeoning of the wildlife industry. Since 2016, there have been outbreaks of African swine fever (ASF) in pigs outside the controlled area that cannot be linked to transfer of infected animals or products from the north. An investigation in 2008–2012 revealed that the presence of *Ornithodoros* ticks and ASFV in warthog burrows extended marginally across the boundary of the controlled area. Furthermore, a recent revision of the taxonomy of Afrotropical *Ornithodoros* ticks included description of new species and indicated that some species were present outside the controlled area.

The present project was undertaken to confirm whether there is sylvatic circulation of ASFV beyond the controlled area through testing warthog sera for presence of antibody and *Ornithodoros* ticks for presence of virus. Although warthogs are widely hunted in South Africa, logistic problems precluded the recruitment of adequate numbers of samples from hunters, and hence opportunistic use was made of samples collected during warthog culling operations in large nature reserves to augment samples obtained from hunters or collected in association with provincial veterinary authorities on farms in the vicinity of past outbreaks of the disease.

The Greater Kruger National Park (GKNP) inside the controlled area was sampled as representative of an environment where circulation of ASFV in ticks and warthogs is endemic, while Addo Elephant National Park (Addo ENP) was included in the study for contrast since the presence of ASFV had never been recorded in the Eastern Cape Province at the time of sampling in 2019, although there were outbreaks of the disease about 250 km east of the national park in the following year. Specific evidence of sylvatic circulation of ASFV was sought in Mokala National Park (Mokala NP) because contact with warthogs had been reported in outbreaks of the disease in domestic pigs in the Northern Cape Province and adjacent western Free State Province. Stored serum samples were also obtained opportunistically from 44 warthogs culled in 2017 in the provincial Rolfontein Nature Reserve (Rolfontein NR), 100km south of Mokala NP. Besides serum for antibody tests and whole blood samples taken with

EDTA anticoagulant, opportunity was taken to collect samples of selected visceral organs and lymph nodes from culled warthogs for determining ASFV presence in warthog tissues to be reported separately from the present project.

Antibody to ASFV was detected by blocking ELISA in 201/207 (97.1%) warthog sera from GKNP, 0/66 sera from Addo NP, 0/44 from Rolfontein NR, 76/77 (98.7%) from Mokala NP, 26/29 (89.6%) dried blood samples from hunted warthogs from properties inside the controlled area and 8/12 (66.7%) warthogs from properties outside the controlled area.

A total of 5078 ticks was collected at 45 locations in 7/9 provinces during 2019–2021 and assayed as 711 pools for virus content by qPCR, while 221 pools were also analysed for tick phylogenetics. Viral nucleic acid was detected in 50 tick pools representing all four members of the *Ornithodoros* (*Ornithodoros*) *moubata* complex known to occur in South Africa: *O. (O.) waterbergensis* and *O. (O.) phacochoerus* species yielded ASFV genotypes XX, XXI, XXII at 4 locations and *O. (O.) moubata* yielded ASFV genotype I at two locations inside the controlled area. Outside the controlled area, *O. (O.) moubata* and *O. (O.) compactus* ticks yielded ASFV genotype I at 7 locations, while genotype III ASFV was identified in *O. (O.) compactus* ticks at a single location. Two of the three species of the eyed *O. (O.) savignyi* complex ticks known to be present in the country, *O. (O.) kalahariensis* and *O. (O.) noorsveldensis*, were collected at single locations and found negative for virus. The only member of the *Pavlovskyella* subgenus of *Ornithodoros* ticks known to occur in South Africa, *O. (P.) zumpti*, was collected from warthog burrows for the first time, in Addo National Park in the Eastern Cape Province where ASFV had never been recorded, and it tested negative for the ASF viral nucleic acid. Since ASF subsequently occurred in Eastern Cape Province, a short communication on the finding of *O. (P.) zumpti* in warthog burrows was published separately to stimulate investigation of the possible involvement of the tick in ongoing outbreaks there.

The current observations extend the known distribution ranges of some tick species and provided presumptive evidence of their role as vectors of ASFV. While it was confirmed that there is sylvatic circulation of ASFV outside the controlled area in South Africa, there is a need for more extensive surveillance and for vector competence studies with various species of *Ornithodoros* ticks, including *O. (P.) zumpti*, and *O. (O.) compactus* that had previously only been known as a parasite of tortoises.

CHAPTER 1

Literature review and motivation



1.1 Introduction

African swine fever (ASF) is a contagious and lethal viral disease of domestic pigs. The virus spreads rapidly in naïve pig populations and may attain fatality rates approaching 100%, but where infection is not eradicated strains with reduced virulence can arise (Penrith et al., 2019). The causative agent, African swine fever virus (ASFV), is presumed to have evolved in eastern and southern African sylvatic circulation between warthogs, and argasid ticks of the genus *Ornithodoros* (Jori and Bastos, 2009; Penrith and Vosloo, 2009). The disease was first recorded in Kenya in 1909 in domestic pigs farmed in proximity to warthogs (*Phacochoerus africanus*) and bushpigs (*Potamochoerus larvatus*) (Montgomery, 1921). In experimental infections, both bushpigs and warthogs underwent benign viraemic infection but were not contagious for domestic pigs (Montgomery, 1921). It was only after the occurrence of the disease had been recognized in South Africa in 1926 (Steyn, 1928) that the virus was recovered from naturally infected warthogs by inoculation of blood subcutaneously in domestic pigs (Steyn 1932). Over the next few decades spread of the disease in Africa was facilitated by a massive increase in small-scale pig farming that frequently involved free-ranging animals and informal trading in communal and peri-urban areas (Penrith et al., 2019).

Multiple genotypes of the virus are known to exist and in 1957 and again in 1960, genotype I was accidentally introduced into the Iberian Peninsula, putatively from western Africa in contaminated swill derived from airline catering. The infection spread in Europe, and to the Caribbean and South America on the second occasion, taking decades to control. Genotype II was introduced into Georgia in 2007, ostensibly from eastern Africa in swill from a ship, and infection spread progressively in Eastern and Central Europe.

The destructive potential of the disease was fully appreciated with the entry of the virus into China, the world's biggest pork producer, in 2018. The disease spread rapidly across China and into other Asian territories (Dixon et al., 2020). China's economic growth and high demand for pork had resulted in an increased number of small-scale farmers. As has been seen in Africa, small-scale farmers are not able to endure the ever-rising cost of commercially-produced feeds. Therefore, locally produced feed is used, in many cases containing potentially infected swill. Informal trading without appropriate biosecurity protocols is also reported. The sheer size of the pork industry in China renders control and prevention measures extremely complex (Dixon et al., 2020).

To date, the virus remains active in many countries, including its re-appearance in the Americas following a near 40-year absence. Losses are far greater abroad than in Africa

where pig farming occurs on a more limited scale. Consequently, ASF is no longer an African disease but an unprecedented global problem.

1.2 Aetiology of ASFV

African swine fever virus is the sole member and type species of the *Asfivirus* genus of the *Asfarviridae* family and only DNA virus known to be arthropod-borne. However, a novel *Asfivirus*-like virus, provisionally designated as abalone asfa-like virus (AbALV), was recently described as the cause of abalone amyotrophia (Dixon et al., 2013; Penrith et al., 2019; Matsuyama et al., 2020).

African swine fever virus is a large, enveloped, double-stranded DNA virus with icosahedral morphology and an external diameter of approximately 200nm (Figure 1.1) (Salas and Andrés, 2013; Penrith et al., 2019). The core nucleoid contains the genome and nucleoproteins. It is coated by a 30 nm protein shell that constitutes about 32% of the total mass of the virion. The core shell is surrounded by an inner single lipid membrane, proposedly derived from the endoplasmic reticulum (Salas and Andrés, 2013). Next there is the capsid formed by approximately 2,000 capsomeres (Salas and Andrés, 2013). The virion outer envelope resembles a plasma membrane and is acquired during virus budding (Salas and Andrés, 2013).

Isolates vary in genomic length between 170 and 193 kilo base pairs (kbp) owing to gains and/or losses of multigene family members in the central conserved region and left or right variable regions (Blasco et al., 1989). Available data indicates a genome comprising 151-167 open reading frames encoding 160 – 175 genes including structural and non-structural proteins, assisting with but not limited to; ontogenesis, replication and evasion of the host cells defence (Figure 1.1) (Kleiboeker et al., 1998, Penrith et al., 2019; Dixon et al., 2019).

1.2.1 Internalization to uncoating

The virus targets mononuclear phagocytes, preferentially monocytes and macrophages (Dixon et al., 2020). Infection of other cells is possible, although with notable reductions in viral growth (Meloni et al., 2022). Several modes of entry have been described which include receptor and/or non-receptor mediated processes (Angulo et al., 1993; Alonso et al., 2013; Sanchez et al., 2017; Gaudreault et al., 2020). The virus exploits fundamental cellular processes such as endocytosis and direct fusion through receptor-dependent mechanisms (Alonso et al., 2013; Dixon et al., 2019; Gaudreault et al., 2020). Many macrophage receptors

have been implicated in viral entry, but no specific receptor has been identified. Endocytosis into clathrin-coated pits has been suggested; a process that allows transport across the plasma membrane in all mammalian cells (Mellman, 1996; Alonso et al., 2013; Sanchez et al., 2017). Non-receptor mediated macropinocytosis, an actin-dependant process, has also been widely investigated. The process involves viral triggering of macropinosomes allowing for introduction into the cytoplasm to form viral factories in the cytosol (Sanchez et al., 2012, 2017; Hernáez et al., 2015; Dixon et al., 2019; Gaudreault et al., 2020). Fc-receptor-mediated cell entry following an intricate macrophage/receptor process has been proposed, mediating ASFV infection of macrophages via IgG antibody-antigen complexes as demonstrated for many viruses, although the process is complex and further investigations are warranted (Sanchez et al., 2017; Sunwoo et al., 2019; Gaudreault et al., 2020).

1.2.2 Viral replication to egress

Replication begins in the nucleus, but is predominantly cytoplasmic, starting soon after entry into host cells (Simões et al., 2019; Gaudreault et al., 2020; Wang et al., 2021). Early gene expression starts at 4-6 hours post infection (hpi), with replication proteins, following which the ASFV genome is replicated at 6-8hpi (Wang et al., 2021). Intermediate expression of new virion structural proteins follows at 8-16hpi. Late expression occurs at 16-24hpi where the virus particles are assembled in areas known as viral factories, following which, new viral particles move toward the plasma membrane for egress via budding at 24hpi, (Jouvenet et al. 2004; Dixon et al., 2013; Penrith et al., 2019; Wang et al., 2021).

1.2.3 African swine fever virus diversity

Eight serotypes have been identified based on virus hemagglutination inhibition assays. Malogolovkin et al., (2015) proposed the genotyping of CD2-like protein (CD2v) and C-type lectin genes as a simplified method to group ASFV into serogroups. To date 24 genotypes (I–XXIV) have been identified based on partial *p72* gene sequencing. Other gene targets have been used for greater resolution of relationships within the genotypes. The following three gene targets have been extensively used in the epidemiology of the disease (WOAH, formerly Office International des Épidémiologies, OIE):

(i) *P72 (B646L)* genotyping

Virus protein 72 (VP72) is the major capsid protein encoded by the *B646L* or *p72* gene and was one of the first targets used for ASFV genotyping. (Tabarés et al., 1980; Lopez-Otin et al. 1990; Yu et al., 1996). Twenty four genotypes (I–XXIV) have been described using partial sequencing of the C-terminus region of this gene. All 24 genotypes are present in Africa (Achenbach et al., 2017; Bastos et al., 2003; Boshoff et al., 2007; Lubisi et al., 2005; Quembo et al., 2018). Only two genotypes, namely genotypes I and II, have been recorded outside the African continent and are responsible for the current global occurrence of the disease (Mulumba-Mfumu et al., 2019). Subtypes within genotype I have been described based on the occurrence of single nucleotide polymorphisms within *p72 (B646L)* gene (Amar et al., 2021).

(ii) *P54 (E183L)* genotyping

Virus protein 54 (VP54) encoded by the *E183L* gene was used to increase intra-genotypic resolution of *p72* genotypes. However, few phylogenetic studies have been conducted in comparison to the *p72* gene. Gallardo et al., (2009) investigated additional strategies to analyse the phylogeny of ASFV isolates in Kenya and Uganda by evaluating *p72* and *p54* simultaneously and reported the phylogenetic analysis of both assays was near identical. During the analysis, four subtypes of genotype I were identified, namely Ia, Ib, Ic, and Id, however this designation should not be confused with the same designations applied to ASFV genotypes defined by *p72* gene sequences (Amar et al., 2021). It is advocated that the *p54* gene should only be used as an auxiliary to other phylogenetic assays (Qu et al., 2022).

(iii) CVR (9RL/*B602L*) genotyping

Identifying differences between viruses of the same *p72* genotype can also be achieved by comparing amino acid sequences of tetrameric repeats from the central variable region (CVR), located within the 9RL open reading frame. Bastos et al., (2004), however, warns that low genetic diversity occurs and CVR alignments become difficult and biased when used alone to evaluate ASFV genotypes. This approach, therefore, should be considered complementary to other genotyping methods.

1.2.4 Alternative analysis

Since CD2v and C-type lectin gene loci mediate serologic specificity they are useful in immunological studies (Malogolovkin et al., 2015) However, next-generation sequencing (NGS) is becoming increasingly available, both in research and clinical settings. This platform although not without its drawbacks, enables a much more comprehensive analysis of ASFV isolate relationships, antigenic diversities, epidemiological tracing, diagnostics and may assist with the identification of potential vaccine candidates (Dixon et al., 2013).

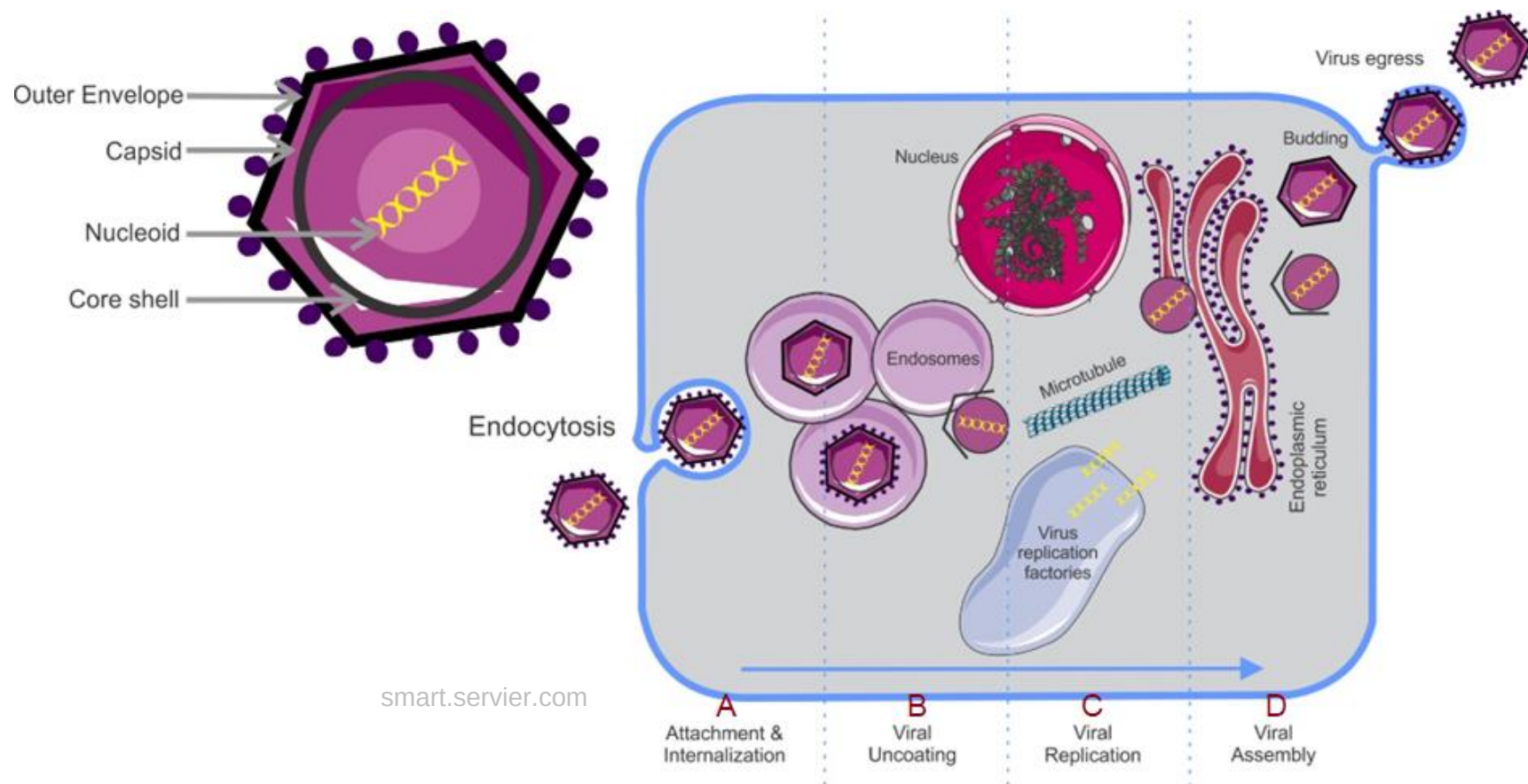


Figure 1.1: Schematic representation of ASFV structure and viral replication. Adapted from Gaudreault et al., (2020). (A) ASFV host cell entry via endocytosis; macropinocytosis; Fc-receptor mediated and/or phagocytosis. (B) Intracellular vesicles are formed composed of adaptor protein coated clathrin producing early endosomes or macropinosomes allowing for viral uncoating. (C) Replication of the ASF viral genome occurs briefly in the nucleus, but is predominantly cytoplasmic, taking place in viral factories. Gene expression initially involves replication proteins followed by the production of structural proteins, allowing for viral assembly. (D) Assembled virions are released via budding.

1.3 Pathogenesis

The present study is principally concerned with the circulation of virus between tick vectors and warthogs, and since the disease presentation and pathogenesis in domestic pigs has been extensively reviewed (Penrith et al., 2019; Salguero, 2020) the subject is only briefly discussed here.

The clinical presentation of ASF infection ranges from peracute to subclinical disease. The disease outcome has been linked to factors such as age, infectious dose and the virulence of the isolate. Virus isolates may be of high, moderate or low virulence (Pan and Hess, 1984). Highly virulent strains cause peracute or acute fatal disease, moderately virulent isolates may cause acute and subacute disease. Chronic forms of the disease have been reported, while low virulence isolates produce mild and non-specific signs easily confused with other diseases (Penrith et al., 2019; Sánchez-Cordón et al., 2021).

1.3.1 Peracute disease

Disease caused by highly virulent ASFV isolates is typically characterized by rapid onset, anorexia, lethargy, and sometimes sudden death with either no clinical signs before death or marked pyrexia and animals rapidly becoming comatose or moribund. This form is often observed when the virus enters naïve populations (Penrith et al., 2019; Penrith and Vosloo, 2009).

1.3.2 Acute disease

Acute disease is caused by virulent isolates. It is associated with clinical signs including but not limited to marked pyrexia, tachycardia and tachypnoea, lethargy, anorexia, and recumbency. Pigs may also exhibit haemorrhage, and/or petechia of the skin. Death follows from 2-13 days after infection and mortality approaches 100% (Penrith et al., 2019; Penrith and Vosloo, 2009).

1.3.3 Subacute disease

Subacute forms of the disease are usually caused by low or moderately virulent isolates and in many cases are altogether subclinical. When clinical signs are apparent, they are generally milder than seen in acute forms and the onset of the disease is usually 2-10 days. Clinical

cases last approximately 20 days and pigs usually succumb to the disease two weeks after infection. Mortality may reach 70% (Penrith et al., 2019).

1.3.4 Chronic disease

In the chronic form of the disease onset may be insidious and clinical signs atypical and persistent. Non-specific signs may include skin ulceration, arthritis, stunted growth, emaciation, pneumonia and abortion. Mortality rates may be low (>30%) (Penrith et al., 2019).

Sub-clinically or chronically infected survivor pigs, can remain persistently infected for months, and long term survival of up to 465 days has been reported. This may contribute to transmission and play an important role in disease persistence in endemic areas, including sporadic outbreaks and ASF introductions into disease-free populations (Penrith and Vosloo, 2009). However, such pigs are not considered subclinical carriers in the traditional sense, as they are unhealthy and eventually die of the disease (Ståhl et al., 2019; Penrith et al., 2019).

1.4 Epidemiological cycles

Historically, three epidemiological transmission cycles have been described for ASF: a sylvatic cycle, a tick-pig cycle and a domestic pig-pig cycle (Costard et al., 2013). However, a fourth transmission cycle involving the wild boar population of Europe has been proposed (Figure 1.2) (Chenais et al., 2018).

1.4.1 Sylvatic Cycle

Infected adult warthogs show no clinical signs of long-term infection; with virus persisting in lymphoid tissues and viraemia is usually undetectable or of low-titre (Thomson, 1985). Horizontal and vertical transmission does not appear to occur between warthogs, and likewise there is no direct transmission from warthogs to domestic pigs. Maintenance of the virus is dependent on the relationship between the ticks that inhabit warthog burrows and the warthog piglets that become infected during the farrowing season (October – December). Warthog piglets develop intense viraemia for several weeks after being bitten by infected ticks in burrows, allowing naïve ticks that take blood meals to become infected and thus perpetuate the transmission cycle (Thomson et al., 1980, Plowright et al., 1994, Costard et al., 2013).

Warthogs can transmit infection to domestic pigs indirectly by conveying infected ticks to the vicinity of piggeries, or else when tissues of infected warthogs are fed to pigs as swill, although the infectivity of warthog tissues for domestic pigs is disputed (Costard et al., 2009, Costard et al., 2013; Penrith et al, 2019).

Bushpigs (*Potamochoerus porcus*) have been shown to develop more intense viremia than warthogs and to be transiently contagious for domestic pigs (Anderson et al., 1998). Since bushpigs do not live in burrows it is surmised that they are not exposed to infection from *Ornithodoros* ticks. Natural infection of bushpigs has been detected in East Africa and Madagascar. It is thought that they become infected through environmental contamination in East Africa and may transmit infection to domestic pigs in the same manner. In South Africa, bushpigs occur in wooded areas in the north of the country and inland from the eastern and southern coastlines but are absent from the more arid northwest and are considered unlikely to play a significant role in infecting domestic pigs (Detray, 1963; Mansvelt, 1963; Thomson et al., 1980; Anderson et al., 1998; Oura et al., 1998; Ravaomanana et al., 2011; Okoth et al., 2013; Ståhl et al., 2014; Kukiela et al., 2016; Salguero, 2020).

Another member of the *Potamochoerus* genus, the red river hog (*P. porcus*), that occurs in rain forests from Senegal to the Congo Basin and, the giant forest hog (*Hylochoerus meinertzhageni*) that occurs in tropical forests of Central Africa have been found infected with ASFV on isolated occasions but the distribution of these species is much more restricted than bushpigs and even more so than warthogs and therefore, they are unlikely to be a regular sources of infection for domestic pigs (Heuschele and Coggins, 1965; Luther et al., 2007).

1.4.2 Domestic pig-tick cycle

A transmission cycle involving domestic pigs and *Ornithodoros* ticks was first reported to occur in the Iberian Peninsula after escape of the virus to Europe. In Africa, ASFV was found in *Ornithodoros* ticks that had become established in the cracks and crevices of rudimentary pig shelters in Malawi, Madagascar and Mozambique (Sánchez Botija, 1963; Haresnape et al., 1988; Ravaomanana et al., 2011; Quembo et al., 2018). It is not apparent from the literature that the domestic pig-tick cycle has been adequately investigated elsewhere in Africa.

1.4.3 Domestic pig-pig cycle

Once ASFV is introduced into a domestic pig population, it is transmitted rapidly by direct contact between infected and susceptible pigs causing high mortality, with a few potential survivors, remaining chronically infected (Costard et al., 2009, 2013). Chronically infected individuals do not usually transmit infection beyond 20-40 days' post infection (de Carvalho Ferreira et al., 2013), unless they are slaughtered and their tissues fed to other pigs in swill. However, where outbreaks are not rigorously controlled by slaughter and re-stocking, ASFV isolates with reduced virulence tend to emerge and persist in pig populations (Penrith et al., 2019).

1.4.4 Eurasian wild boar-habitat cycle

Both direct transmission between infected and susceptible wild boars and indirect transmission through contamination of the habitat by infected carcasses occur in Eastern Europe, with the cold climate facilitating long term survival of virus in carcasses (Chenais et al., 2018). This cycle is currently considered one of the main drivers of persistent infection in the Caucasus, Russia, and the Baltic States, posing a major threat to the European pig industry (Guberti et. al., 2019).

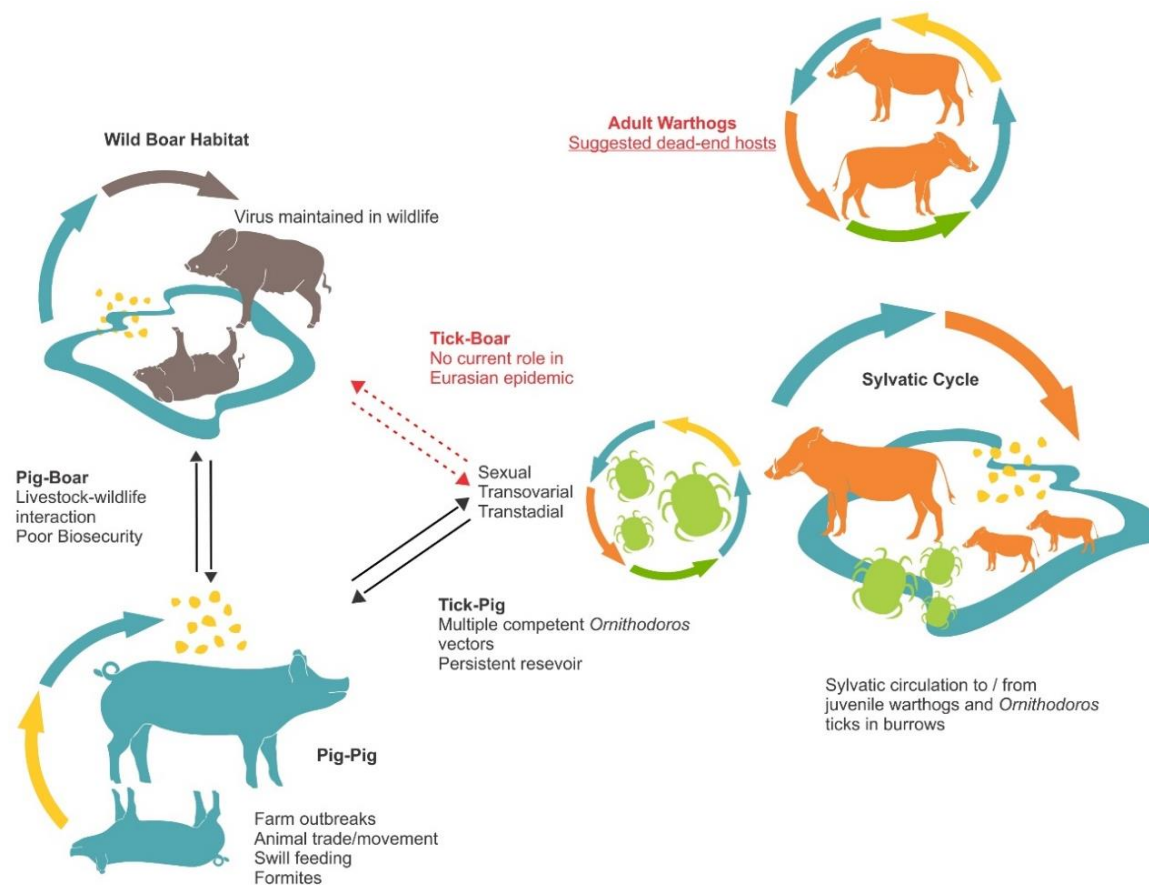


Figure 1.2: ASFV transmission cycle schematic; Adapted from Gaudreault et al., (2020). In Eurasia and Africa, ASFV is commonly transmitted between domestic pigs through direct contact, consumption of contaminated pig products and through fomites (Pig-Pig). In Eurasia, transmission between domestic pigs and wild boars takes place at the livestock-wildlife interface, linked to breaks in biosecurity. ASF circulation occurs within wild boar populations via indirect environmental contamination (wild boar habitat). ASFV can be transmitted between soft ticks of the genus *Ornithodoros* established in domestic pig enclosures, therefore serving as persistent reservoirs of ASFV as seen in the Iberian Peninsula, East and Southern Africa (Pig-Tick cycle). No interactions between wild boar and soft ticks has been described. Sylvatic circulation in Eastern and Southern Africa involves virus transmission between warthog (*Phacochoerus africanus*) piglets and *Ornithodoros* ticks. Warthogs piglets develop intense viremia facilitating infection of naïve ticks which feed on them. Adult, warthogs undergo long-lasting infection of lymphoid tissues and are considered dead-end hosts. *Ornithodoros* tick vectors are able to transmit the virus transstadially, sexually, and transovarially.

1.5 *Ornithodoros* tick vectors

Ticks of the genus *Ornithodoros* within the family *Argasidae* have a near global distribution, almost all occurring in tropical and/or sub-tropical habitats and comprise over 100 species; the exact number varying with periodic discoveries and taxonomic revisions (Taylor et al., 2007; Bakkes et al., 2018; Mans et al., 2019). In South Africa, argasids are colloquially referred to as ‘tampans’. Their waxy cuticle confers resistance to desiccation, facilitating their occurrence in warmer and the more arid regions (Figure 1.3) (Hafez et al., 1970; Vial, 2009; Kaufman, 2013). Male and female soft ticks are similar in appearance and sexual dimorphism is only visible in adults that can be distinguished by the shape of the genital aperture.

The ticks are nidicolous, living in close association with their hosts with distinction made between endophilous nidicoles such as eyeless *Ornithodoros moubata* sensu lato ticks that live within host habitations including animal burrows, and harbourage nidicoles, such as eyed *Ornithodoros savignyi* s.l. ticks that live in the environs of their hosts, such as sand at animal resting sites above ground (Vial, 2009; Gray et al., 2013).

The type species of the genus *Ornithodoros*, *O. savignyi* (Audouin, 1827), was originally described as *Argas savignyi* Audouin, 1827 from specimens collected from sand in Egypt. The name *Ornithodoros moubata* (Murray) (1877), sensu Walton, 1962, was originally applied as *Argas moubata*, Murray, 1877 to a tick found in Angola. Both species were subsequently transferred to the genus *Ornithodoros* that was erected by Koch, (1884). Many authors simply distinguish between eyeless ticks of the *O. (O.) moubata* complex and eyed ticks of the *O. (O.) savignyi* complex, but the ticks of the *Ornithodoros* subgenus have a complex taxonomic history culminating in the recent revision of the subgenus including recognition of new species, as discussed in detail in Chapter 4.

Unlike ixodids, larval ticks of the subgenus *Ornithodoros* tend to moult directly into the first nymphal instar without feeding and nymphs undergo 2–8 moults depending on species and adequacy of blood meals. Furthermore, argasid ticks including *Ornithodoros* species generally feed rapidly in minutes to hours, and leave their hosts to moult to the succeeding instar within weeks to months or to lay eggs as adult females (Vial, 2009; Gray et al., 2013).

The involvement of arthropods in transmission of ASFV was considered a possibility at an early stage in the investigation of the disease (Montgomery, 1921). However, transmission of the virus by *Ornithodoros* ticks of the *Pavlovskyella* subgenus was only recognized after the virus had been introduced into Spain (Sánchez Botija, 1963). Transmission of ASFV by

Ornithodoros ticks was subsequently confirmed in Africa (Plowright et al., 1969a; Plowright et al., 1969b). The life cycles of *Ornithodoros* ticks and their relationships to warthogs and ASFV are discussed in Chapter 4.

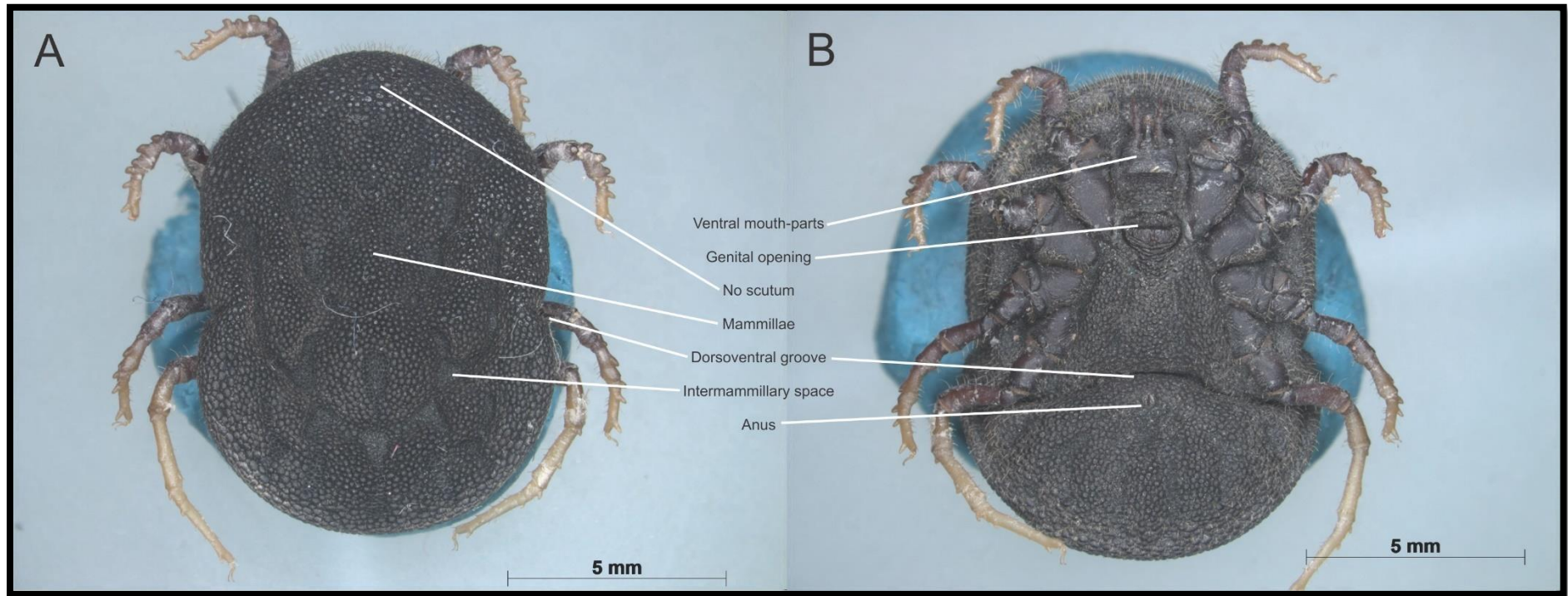


Figure 1.3: Prominent morphological features of an adult *Ornithodoros* (*Ornithodoros*) spp. Female, (A) Dorsal view (B) Ventral view

1.6 Diagnosis of ASFV infection

Methods for diagnosis of ASFV infection currently approved by WOAH (OIE) include detection of viral nucleic acid by conventional or quantitative PCR, isolation of virus in monocyte or macrophage cultures prepared from peripheral blood leucocytes or bone marrow of pigs, or detection of viral antigen by immunofluorescence or enzyme-linked immunosorbent assays (ELISA). The PCR tests are more sensitive than isolation of virus, provide rapid diagnoses and can be used in conjunction with sequencing for identification of virus genotypes. Immune responses can be detected by blocking ELISA (kits are commercially available), indirect immunofluorescence, indirect immunoperoxidase test, or immunoblotting. Rapid pen-side diagnostic tests and the use of cell lines in place of primary pig cell cultures have been investigated and while none have yet been approved by WOAH, portable next generation sequencing devices could find diagnostic and epidemiological application (Sánchez-Vizcaíno and Heath, 2019; Penrith et al., 2019; Gaudreault et al., 2020; Pikalo et al., 2021).

1.7 African swine fever in South Africa

Outbreaks of disease similar to the then newly-described ASF in Kenya, were reported in 1926 in pigs farmed in the northernmost Limpopo Province of South Africa, and it was found that pigs could be infected by inoculation with blood from warthogs shot in the district involved (Steyn, 1928, 1932). Outbreaks of the disease elsewhere were caused by movement of infected pigs or pork products from the north, notably to farms in the environs of Johannesburg in Gauteng Province and from there to properties in Western Cape Province. Consequently, a controlled area (Figure 1.4) was declared in the north and regulations were implemented to prevent transfer of infected suids or products to the rest of the country (De Kock et al., 1940). The northern most tip of KwaZulu-Natal Province was included in the controlled area and the presence of the sylvatic cycle was later confirmed there in Mkhuze Game Reserve (Thomson et al., 1983). However, no outbreaks of ASF have been recorded in that province. No ASFV was found in ticks collected in Mkhuze Game Reserve in 2002, and more recently no ticks were found in warthog burrows in the nearby Ndumo Game Reserve (Arnot et al., 2009; Mapendere et al., 2021).

It was not until 1939 that the last outbreak of the disease outside the controlled area was eradicated in Western Cape Province. Apart from isolated outbreaks in 1951 associated with swill from abattoirs in Gauteng Province, one of which had received infected pigs from Namibia, no further cases of ASF were reported outside the controlled area until 1996, when pigs on 3 farms

close to the boundary of the controlled area in northern Gauteng Province were suspected of acquiring infection from contact with warthogs and/or ticks (Neitz, 1963; Penrith and Vosloo, 2009; Penrith et al., 2013). Thereafter, the regulatory measures proved effective until 2012, when a series of outbreaks on smallholdings in adjacent areas of Gauteng Province and Mpumalanga Province were traced to illegal movement of infected pigs from Limpopo Province followed by local spread of infection involving sale of animals at auctions (Geertsma et al., 2012; Magadla et al., 2016; Penrith et al., 2013, Jansen van Rensburg et al., 2020a, b; Amar et al., 2021). However, from 2016 onwards, there were successive series of outbreaks of ASF in the Gauteng, Mpumalanga, North West, Northern Cape and Free State provinces that could not be linked to movement of infected pigs or products from the controlled area (DALRRD, 1993-2019; Janse van Rensburg et al., 2020a; Mulumba-Mfumu et al., 2019; WAHIS, 2005-2020).

A structured serosurvey found that the outbreaks of 2016 and 2017 had been eradicated effectively, with no evidence of persistence of infection in local pig populations (Janse van Rensburg et al., 2020a, c). Nevertheless, outbreaks continued to occur outside the controlled area, and currently there are three active outbreak events. The first, beginning in 2019, has affected the Free State, Gauteng, Mpumalanga, and North West provinces. The second event constitutes the first known outbreak of ASF in the Eastern Cape Province and started in 2020. The third event, involving the Western Cape Province represents the first outbreak in the province since the disease was eradicated in 1939 (DALRRD 2020, 2021; Janse van Rensburg et al. 2020a, b, c; Amar et al., 2021). Ten of the known 24 genotypes have been identified in South Africa based on *p72* sequencing (I, II, III, IV, VII, VIII, XIX, XX, XXI and XXII) (Boshoff et al., 2007; DALRRD, 2021; Janse van Rensburg et al. 2020a, b; Amar et al., 2021; Mushagalusa et al., 2021; Craig et al., 2022). Two genotypes (I and II) have been identified as drivers of the outbreaks south of the controlled area in domestic pig populations.

Outbreaks of ASF are notifiable to WOA (OIE) and are subject to compulsory control in South Africa by the Department of Agriculture, Land Reform and Rural Development (DALRRD) through State Veterinarians of the Provincial Veterinary Services. The controlled area and ASF regulations promulgated in 1935 have been amended over the years and currently Section 31 of the Animal Diseases Act (Act 35 of 1984) and the associated Animal Disease Regulations apply as set out in the DALRRD Veterinary Procedural Notice for African Swine Fever Control in South Africa of 2018. Although Act 35 of 1984 has been repealed and replaced, Section 31 and certain other sections remain in force. Essentially, the control measures consist of supervised slaughter and safe disposal of infected pig herds, disinfection of premises and re-stocking with disease free

animals after a prescribed interval that varies with conditions on the property involved. Pig farming and disposal of live animals or products in the controlled area are also subject to regulation. Carcasses of hunted or culled wild suids may be transported from the property of origin under veterinary permit provided that the skin, head, hooves and viscera with lymph nodes have been removed.

The ASF controlled area as delimited in 1935 corresponded to the known distribution range of warthogs in South Africa at the time (De Kock et al., 1940), but since then there have been major translocations of warthogs to the south of the country (Figure 1.5). Legal changes relating to wildlife utilization and land ownership encouraged the growth of an extensive wildlife industry based on state-owned and private nature reserves plus game ranches that generate income from tourism, hunting and live animal sales (Benson, 1991; Barnes and De Jager, 1996; Castley et al., 2001; van der Merwe and Saayman, 2003; Lindsey et al., 2013; Swanepoel, 2016). Translocations usually represent reintroduction of indigenous species into sites where they had become locally extinct, but in the instance of the common warthog (*P. africanus*) it has been introduced into the southern parts of South Africa considered to be the former range of the extinct Cape warthog (*P. aethiopicus*) (Swanepoel, 2016).

Initial translocations were conducted by the South African National Parks Board (SANParks) and involved transfer of warthogs considered to be free of ASF from the Hluhluwe-iMfolozi Game Reserve complex in KwaZulu-Natal Province to the Golden Gate National Park in Free State Province in 1963 and 1968 (Penzhorn, 1971), and from the same source to the Andries Vosloo Kudu Reserve (AVKR) (now incorporated into the Great Fish River Nature Reserve) in the Eastern Cape Province in 1976 and 1977 (Somers, 1994). In 1984, warthogs were translocated from AVKR to Rolfontein Nature Reserve in the Northern Cape Province (unpublished records cited by Swanepoel, 2016). Since then there have been numerous unrecorded introductions on a continuing basis into private game reserves and ranches in the above provinces as well as within KwaZulu-Natal Province (Spear and Chown, 2009; Maciejewski and Kerley, 2014).

Warthogs are primarily grazers and have been introduced into favourable areas, often lacking predators and with water and food crops readily available. They are non-territorial and non-migratory, but are difficult to contain by wire, netting or electric fences. Consequently, family groups may have overlapping home ranges that extend over adjacent farms and nature reserves. They have a high reproductive capacity and annual population growth rates. There is breeding dispersal of young animals to neighbouring groups, and as population densities increase at sites

of introduction individuals or groups move to establish new populations. Continued translocations add propagule pressure and hybrid vigour. Hunting contributes to dispersal (Somers and Penzhorn, 1992; Muwanika et al., 2003; Cumming, 2005; Swanepoel, 2016).

Thus warthogs are widely considered to be an invasive species but are valued for tourism and commercial hunting (Swanepoel, 2016). There are no recent estimates of total warthog populations in southern Africa and counts of animals owned or hunted are commonly regarded as intellectual property. It was made known by one organization that 3,849 warthogs were shot by international trophy hunters in 2013 (Professional Hunters Association of South Africa, 2014). Possibly several times that number of warthogs are hunted annually for meat by local clients or culled by landowners to control crop damage and by conservationists for population control in nature reserves and game farms (Swanepoel, 2016).

In a survey conducted on farms up to 20 km north and south of the boundary of the controlled area in 2008-2012, *Ornithodoros* ticks were found in warthog burrows marginally south of the controlled area, including ASFV-infected ticks in one instance. It was concluded that there was no need as yet to realign the boundary of the controlled area, but continued surveillance was advised (Magadla et al., 2016)

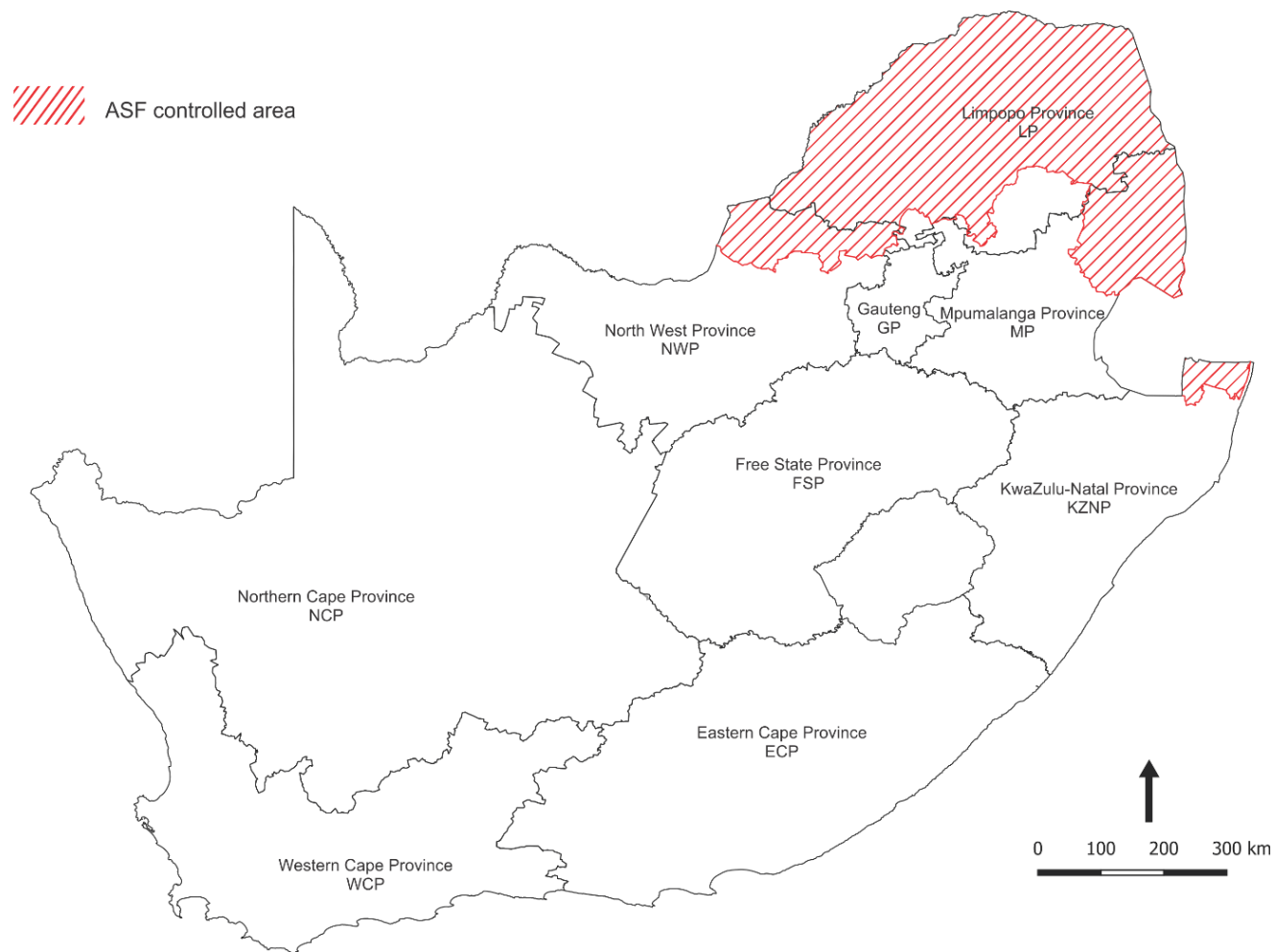


Figure 1.4: African swine fever controlled area within South Africa

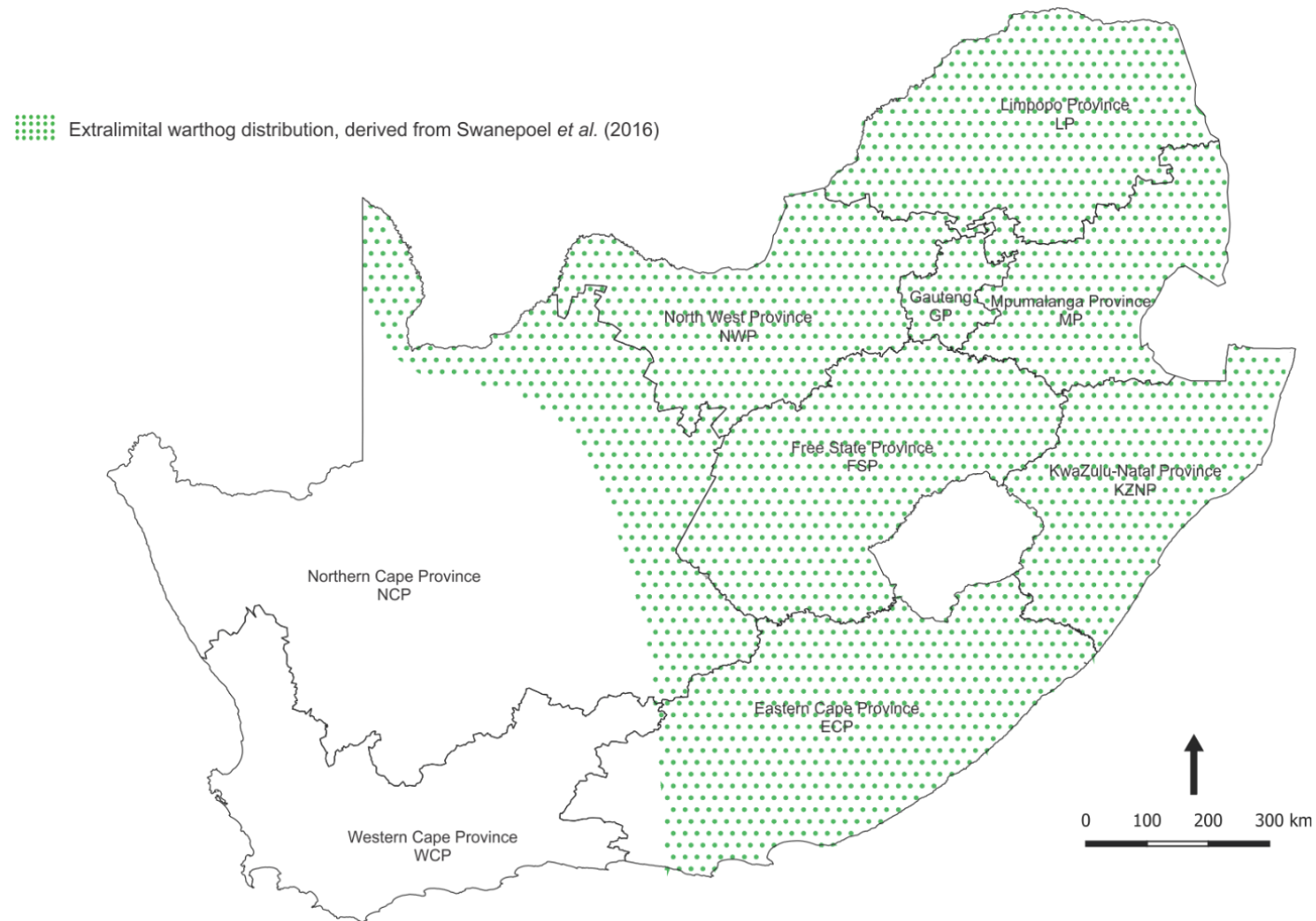


Figure 1.5: Distribution records for common warthog (*Phacochoerus africanus*) (Derived from Swanepoel et al., 2016).

1.8 Motivation and development of project strategy

There was clearly a need to determine whether there was sylvatic circulation of ASFV in warthogs and *Ornithodoros* ticks in locations where outbreaks of disease had occurred south of the controlled area in South Africa during recent years. Accordingly, it was decided to test serum samples from extralimital warthogs for presence of antibody to ASFV with commercially available INgezim PPA Compac R.11.PPA.K3 kits (Eurofins Technologies Ingenasa, Madrid, Spain). The kits are based on a blocking ELISA, so theoretically they can be used to detect antibody activity to ASFV in the serum of any species. Nevertheless, since the tests are not accredited for use on warthog sera, positive findings were to be supported by detection of virus in ticks collected from warthog burrows on properties where antibody was found. Moreover, the tests would be applied to samples collected on properties inside the controlled area where sylvatic circulation of virus is known to be endemic as well as to samples from the broader zone south of the controlled area where the presence of extralimital warthogs had been confirmed in recent years (Figure 1.5) (Swanepoel et al., 2016), but where the presence or absence of the virus was speculative.

Since definitive determination of the prevalence and distribution of antibody in extralimital warthogs lay beyond the resources and time available to a postgraduate research project, a sampling procedure was designed to provide a yes or no answer to the occurrence of sylvatic circulation of ASFV outside the controlled area. The findings would determine whether broader surveillance by provincial veterinary services was warranted.

For calculation of the samples required to establish the presence or absence of antibody to ASFV at 95% confidence level, a conservative estimate of the expected detection rate of $\geq 10\%$ was assumed for the presence of ASFV antibody within the controlled area and a minimal expected detection rate of $\leq 1\%$ for the presence of ASFV antibody outside the controlled area.

Using the equation for sampling of a large (infinite) population (Dohoo et al., 2003), $n = \ln \alpha / \ln q$, where n = required sample size, $\alpha = 1 - \text{confidence level}$ ($= 0.05$) and $q = 1 - \text{prevalence}$, the samples required for the two defined areas were determined to be one serum sample for each of 30 random locations inside the controlled area, and one serum sample for each of 300 locations outside the controlled area (locations were generated with QGIS random point selection). Samples recruited from the field as described below would be assigned to the random geographic point closest to the location of origin of the sample.

Animal research projects require approval by the University of Pretoria Animal Ethics Committee and the Faculty of Veterinary Science Research Ethics Committee, plus permits from DALRRD in terms of Section 20 of the Animal Diseases Act (Act 35 of 1984). Neither the University of Pretoria nor DALRRD allows disclosure of names of owners, or names and addresses or accurate locations of properties, and this information is also covered by the South African Protection of Personal Information Act 4 of 2013. The data has to be anonymized. Furthermore, it was not permissible to solicit owners for consent to capture, immobilize or euthanize wild animals in order to obtain samples for research projects.

However, many public institutions or companies, such as national and provincial nature reserves, openly support hosting of duly registered academic research projects and even require acknowledgement of the organization's participation when findings are made known in conference presentations and scientific publications.

Hunters, businesses that arrange hunts for clients (hunting outfitters) and game ranch owners are represented by several associations in South Africa. It seemed logical to recruit blood samples from hunted warthogs through the associations representing the hunting industry, but a simple and approved method was required for collection of the samples. Dried blood samples collected on filter paper or Nobuto cellulose strips (Advantec, Tokyo, Japan) have been used successfully in antibody surveys on a wide range of wildlife species, as for example on waterfowl for Avian Influenza studies (Dusek et al., 2011). Accordingly, Dr Livio Heath of the Agricultural Research Council-Onderstepoort Veterinary Research Transboundary Animal Diseases Laboratory (TADL) arranged for a small feasibility study to be conducted by his staff, in which a few pig blood samples known to positive or negative for the presence or absence of antibody to ASFV were adsorbed to Nobuto strips, dried, eluted and successfully tested.

Discussions with the University ethics committees established that it would be acceptable to approach land owners (game owners) or their duly authorised representatives (farm managers or supervisors of hunting activities) through associations representing their interests, for access to blood samples collected on Nobuto strips from hunted or culled animals. The associations would approach their members online with documents prepared by the investigator and comprising a one-page description of the project in plain language plus an approved informed consent form (Appendices D and E) to be signed by participating land owners or authorised representatives. An amendment to the Section 20 permit for the project was obtained from DALRRD to allow collection of dried blood samples from warthogs by hunters and for transport of the samples in appropriate biosafety packaging to the Animal

BSL3 facility at TADL to be covered by the veterinary transport permit issued for removal of the hunted carcass, and for conveyance of the samples to be facilitated through the network of the hunting association involved. Samples were to be accompanied by a duly signed consent form and information including the number of the veterinary transport permit, the coordinates of the property concerned, the date of sampling, plus gender and estimated age group of the warthog. Although the project was concerned specifically with warthogs, it was deemed logical to obtain clearance to include other wild suids that may be hunted, namely, feral domestic pigs, bushpigs, and Eurasian wild boars (*Sus scrofa ferus*).

Nobuto strips for collection of samples, appropriate biosafety packaging, project information sheets, consent forms and instruction sheets for taking and onward transmission of samples (Appendices D, E and F) were to be provided by the investigator to hunting associations involved for distribution to participating members.

In preliminary discussions to establish the feasibility of collecting blood samples from hunted warthogs, some organizations indicated reluctance to participate but the largest association representing hunters and game owners in South Africa agreed to cooperate. They canvassed their members and initial expressions of interest gave cause for optimism that the projected sampling requirements would be met. However, logistic problems and hesitancy to participate soon emerged and it became clear that a change in strategy was required.

A more targeted approach to sampling was adopted with emphasis on culling operations in national parks and other nature reserves, and with appropriate amendment of the Section 20 permit, registration of research projects with parks authorities and securing of requisite materials transfer agreements (Appendices C and G). The Greater Kruger National Park (GKNP) inside the controlled area represented an environment where sylvatic circulation of ASFV is endemic, Addo Elephant National Park (Addo ENP) provided contrast since the presence of ASFV had never been recorded in the Eastern Cape Province at the time of sampling, while Mokala National Park (Mokala NP) was included specifically because contact with warthogs had been suspected in outbreaks of the disease in domestic pigs in Northern Cape Province and adjacent western Free State Province. Emphasis was on collection of blood samples for the serosurvey, but opportunity was taken to collect samples of selected visceral organs and lymph nodes from culled warthogs for determining prevalence of ASFV in warthog tissues, to be reported separately from the present project (Figure 1.6). In addition, provincial veterinary officials assisted with access for collection of samples on a few farms in the vicinity of past outbreaks disease in domestic pigs. Tests for antibody to ASFV were also

performed on a limited number of serum and blood samples from spotted hyaenas (*Crocuta crocuta*) and leopard tortoises (*Stigmochelys pardalis*) as alternative vertebrate hosts of *Ornithodoros* ticks. Collection of ticks was performed in the national parks and on numerous other properties, including many where warthog serum samples were not available, and the project incidentally provided additional information on distribution ranges and potential involvement of some species as vectors of ASFV (Figure 1.7).

Description of the field and laboratory studies, plus outcomes and evaluation of the findings are presented in Chapters 2, 3 and 4 which represent manuscripts accepted and published in peer-reviewed scientific journals. The findings are briefly collated and evaluated in the concluding Chapter 5.



Figure 1.6: (A) Collection of blood and selected visceral organ and lymph node samples from culled warthogs. (B) Sampling instruments and containers. Sampling team consisted of study supervisors, the research student, a postgraduate colleague and two undergraduate veterinary research elective volunteers, in association with state veterinary officials.



Figure 1.7: Collection of *Ornithodoros* ticks. (A) Armed game guards required in nature reserves. (B) Removal of soil from burrows in search of *Ornithodoros* ticks (C) Soil content sifted into sorting trays for collection of *Ornithodoros* ticks and (D) *Ornithodoros* tick collection.

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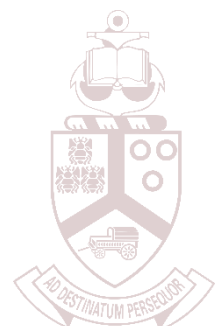
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CHAPTER 2

**Updated distribution and host records
for the argasid tick *Ornithodoros*
(*Pavlovskyella*) *zumpti*: A potential
vector of African swine fever virus in
South Africa**



JOURNAL INFORMATION

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African swine fever virus (ASFV) causes a lethal and contagious disease of domestic pigs. In South Africa, the virus historically circulated in warthogs and ornithodorid ticks that were only found in warthog burrows in the north of the country. Regulations implemented in 1935 to prevent transfer of infected animals or products to the south initially proved effective but from 2016 there have been outbreaks of disease in the south that cannot be traced to transfer of infection from the north. From 1963 there were widespread translocations of warthogs to the south, initially from a source considered to be free of ornithodorid ticks. We undertook to determine whether sylvatic circulation of ASFV occurs in the south, including identification of potential new vectors, through testing extralimital warthogs for antibody and ticks for virus. Results of testing warthogs for antibody and other species of ticks for virus will be presented separately. Here we report finding *Ornithodoros (Pavlovskyella) zumpti* ticks in warthog burrows for the first time. This occurred in the Eastern Cape Province (ECP) in 2019. Since African swine fever was recognised in the ECP for the first time in 2020 and outbreaks of the disease in domestic pigs continue to occur there, priority should be given to determining the distribution range and vector potential of *O. (P.) zumpti* for ASFV.

Keywords: African swine fever virus; sylvatic circulation; arbovirus; South Africa; extralimital warthogs.

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Updated distribution and host records for the argasid tick *Ornithodoros (Pavlovskyella) zumpti*: a potential vector of African swine fever virus in South Africa.

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2.1 Abstract

African swine fever virus (ASFV) causes a lethal and contagious disease of domestic pigs. In South Africa, the virus historically circulated in warthogs and ornithodorid ticks that were only found in warthog burrows in the north of the country. Regulations implemented in 1935 to prevent transfer of infected animals or products to the south initially proved effective, but from 2016 there have been outbreaks of disease in the south that cannot be traced to transfer of infection from the north. From 1963 there were widespread translocations of warthogs to the south, initially from a source considered to be free of ornithodorid ticks and ASFV.

We undertook to determine whether sylvatic circulation of ASFV occurs in the south, including identification of potential new vectors, through testing extralimital warthogs for antibody and ticks for virus.

Results of testing warthogs for antibody and other species of ticks for virus will be presented separately. Here we report finding *Ornithodoros (Pavlovskyella) zumpti* ticks in warthog burrows for the first time. This occurred in the Eastern Cape Province (ECP) in 2019. Since African swine fever was recognized in the ECP for the first time in 2020 and outbreaks of the disease in domestic pigs continue to occur there, priority should be given to determining the distribution range and vector potential of *O. (P.) zumpti* for ASFV.

2.2 Research communication

The argasid tick *Ornithodoros (Pavlovskyella) zumpti* was described in 1953 as a new species from the burrow of a four-striped field mouse (*Rhabdomys pumilio* sensu lato) in the Cathcart district of the Eastern Cape Province (ECP) of South Africa (Heisch and Guggisberg, 1953). In 1961, the same species was collected approximately 300 km south-west of the type locality from the nest of a Saunders' vlei rat (*Otomys saundersiae*) in Kariega district, formerly Uitenhage, ECP, during investigation of the bionomics of the tick (Fearnhead, 1962). More recently, in 2014, *O. (P.) zumpti* was again found in Cathcart district, in a porcupine (*Hystrix africaeaustralis*) burrow, and also in the burrow of a springhare (*Pedetes capensis*) in Makhanda district, formerly Grahamstown, during a phylogenetic study of the *Argasidae* (Figure 2.1) (Mans et al. 2019).

Argasid ticks of the genus *Ornithodoros* serve as biological vectors of African swine fever virus (ASFV) that causes lethal disease in domestic pigs. The virus is maintained in the savannah areas of eastern and southern Africa in sylvatic circulation between warthogs (*Phaecochoerus africanus*) that develop benign viremic infection and eyeless argasid ticks of the *Ornithodoros (Ornithodoros) moubata* complex that live in warthog burrows (Penrith et al., 2019). Warthogs develop intense viremia after infection by ticks while still confined to their burrows as young piglets, but naïve adults develop viremia of low intensity after infection and have not been shown to be contagious for domestic pigs. However, they can convey infected ticks to the environs of piggeries, or infected warthog offal may be fed to pigs in swill. The infection is contagious in domestic pigs and spreads without need for the tick vectors (Penrith et al., 2019).

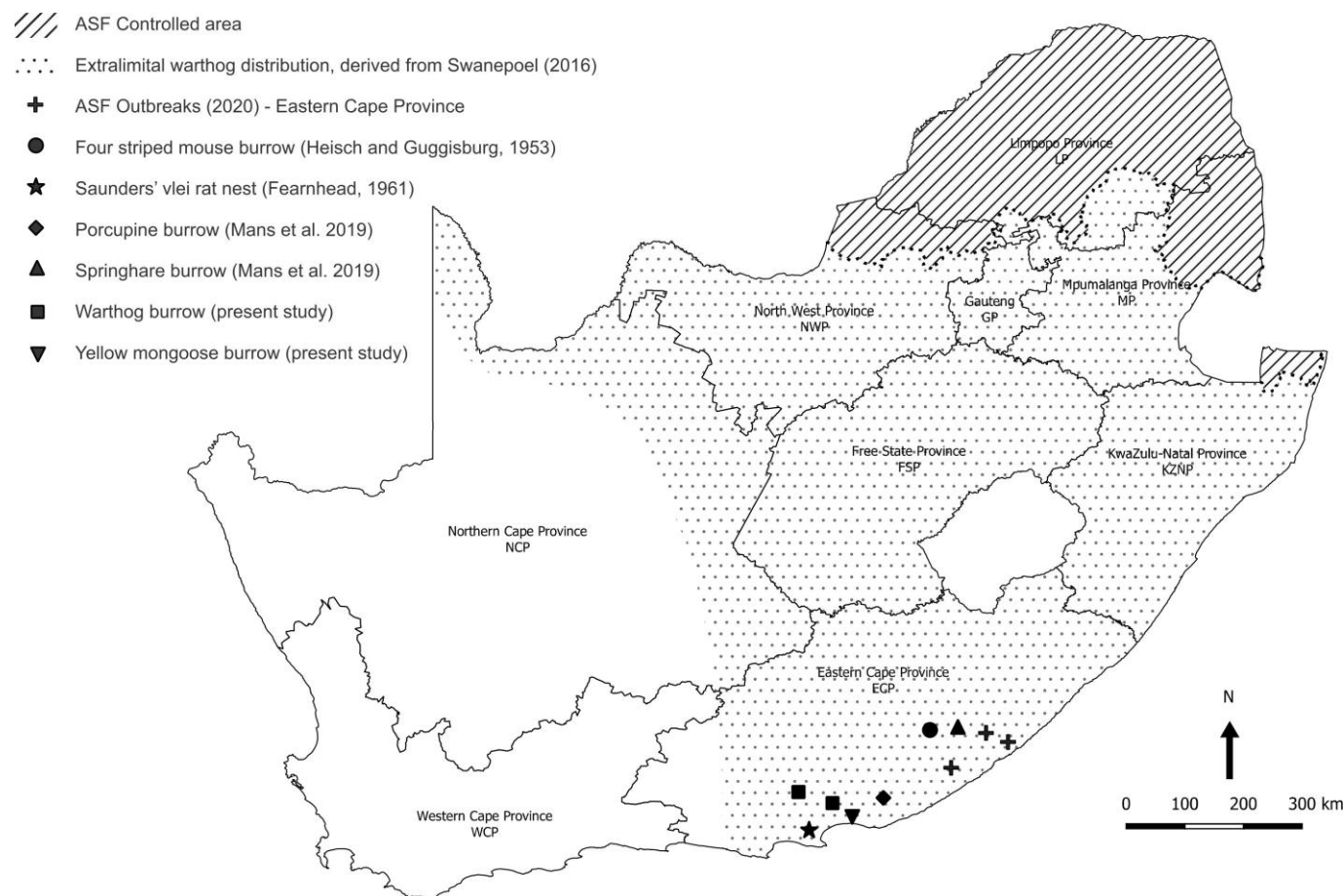


Figure 2.1: Map showing the African swine fever (ASF) controlled area in South Africa, the distribution of extralimital warthogs derived from Swanepoel (2016), the sites of ASF outbreaks in domestic pigs in the Eastern Cape Province in 2020, and the sites of collection of *Ornithodoros (Pavloskyella) zumpti* ticks from host-associated sites as indicated.

Vector-borne transmission of ASFV was first recognized in domestic pigs and *Ornithodoros (Pavlovskyella) erraticus* ticks in Spain after the virus had accidentally been introduced into Europe (Sanchez Botija 1963). Over recent decades both pig farming and the occurrence of ASFV have spread from east to west across Africa (Penrith et al., 2019), and in 2006 the virus was found in *O. (P.) sonrai* ticks collected in rodent burrows close to piggeries in Senegal although the epidemiological significance of the finding was regarded as uncertain (Vial et al. 2007). Only members of the subgenus *Ornithodoros* have been identified as vectors of ASFV elsewhere in sub-Saharan Africa. Nevertheless, vector competence studies in various countries abroad have established that the ability to transmit ASFV is a general property of ticks of both the *Ornithodoros* and *Pavlovskyella* subgenera, but the efficiency of transmission varies with virus isolate and species of tick (Pereira De Oliveira et al., 2020).

In South Africa, ASF was originally prevalent in the northern part of the country where domestic pigs had contact with warthogs (Penrith et al., 2019). Hence, a controlled area was declared in 1935 to include the known distribution range of warthogs at the time (Figure 2.1), and regulations were implemented to prevent transfer of infected suids or products to the south (de Kock 1940). The regulations initially proved effective, but from 2016 onwards there have been a series of ASF outbreaks in domestic pigs south of the controlled area that cannot be linked to transfer of infection from the north, culminating in 2020 in the first known outbreaks of ASF in the ECP, and in 2021 in the first outbreaks in Western Cape Province since the disease was eradicated there in 1939 (Janse van Rensburg et al., 2020; DALRRD, 2020; 2021).

From 1963 onwards, there were widespread translocations of warthogs from the north to nature reserves and game ranches in the southern half of South Africa where the extralimital animals flourished to become an invasive species (Figure 2.1) (Swanepoel, 2016). Originally, the warthogs were obtained by officials of national and provincial parks from Hluhluwe-iMfolozi Game Reserve considered to be free of ornithodorid ticks and ASFV, but it has been suggested that later there were multiple unrecorded translocations from other sources to private nature reserves (Swanepoel, 2016). Further, the taxonomy of the Afrotropical members of the subgenus *Ornithodoros* was recently revised, including description of new species (Bakkes et al., 2018). Although information on the distribution ranges and vector capacity of the ticks remains incomplete, the implication is that the potential exists for sylvatic circulation of ASFV in ornithodorids and extralimital warthogs to occur beyond the controlled area in South Africa. We conducted a survey for evidence of ASFV infection in warthogs and ticks outside the controlled area, to be reported separately, and in the process encountered new hosts for *O. (P.) zumpti* ticks.

We collected ornithodorid ticks identified as *O. (P.) zumpti* from warthog burrows in Addo Elephant National Park (Addo ENP), ECP, in 2019 before ASF had been recorded in the province and found no ASFV nucleic acid in the ticks. Approximately 10% w/v suspensions were prepared by homogenizing pools of 1-2 adults or 5-25 nymphal ticks in phosphate-buffered saline, pH7.2, and automated nucleic acid extraction was performed with IndiMag Pathogen kits (Indical Bioscience, Leipzig, Germany) using slight modifications to the manufacturer's instruction. 200µL tick homogenate supernatant was added to 200µL AL buffer (Qiagen, Dusseldorf, Germany), incubated at 70°C for 10 min and 200µL of the AL lysate added to the extraction buffer. Eluates were tested for ASFV nucleic acid using the real-time quantitative PCR (qPCR) assay of Zsak et al. (2005) as modified by Sunwoo et al. (2019). Positive and no template controls were included in each PCR run.

The ticks were collected in the Nyathi (33.25070S, 24.29166E), Main Camp (33.44492S, 25.78378E) and Colchester (33.41170S, 25.78466E) sections of the park from burrows observed over the course of a week to be occupied by warthogs. The same species was also obtained from a small burrow in Main Camp section (33.44492S, 25.78378E) observed to be occupied by a family of yellow mongooses (*Cynictis penicillata*) (Figure 2.1).

Identification of the ticks was based on morphology (Heisch and Guggisberg, 1953), confirmed by the Gertrud Theiler Tick Museum of the Agricultural Research Council-Onderstepoort Veterinary Research, Onderstepoort, South Africa, and by amplification and comparison of partial tick mitochondrial 16s rRNA gene sequences with GenBank data (Figure 2.2) (Bakkes et al., 2018; Mans et al., 2019). The *O. (P.) zumpti* sequences fell into 3 clades with 94-96% identity, and more extensive sequencing studies are required to determine whether the differences represent within-species variation or novel cryptic species.

Partial cytochrome B sequences of mammalian mitochondrial DNA were determined from suspensions of partially engorged ticks (Lah et al., 2015), and compared with sequences in GenBank (NCBI) using a BLASTx search to confirm warthogs as blood meal donors for *O. (P.) zumpti*. Insufficient nucleic acid of ticks from the mongoose burrow remained available for cytochrome B analysis.

The morphology of *O. (P.) zumpti* was well described by Heisch and Guggisberg (1953), but since it is presently the only known representative of the subgenus *Pavlovskyella* in South Africa preliminary identification can be based on macroscopic features in the field. In each instar of their life cycle *O. (P.) zumpti* ticks are noticeably smaller than members of the *Ornithodoros* subgenus that occur in warthog burrows. From a dorsal view, ticks of the *Ornithodoros* subgenus have a broadly rounded shape at both the anterior and posterior ends

and are slightly concave bilaterally. *O. (P.) zumpti* ticks have a bluntly pointed hood that covers the basis capituli at the anterior end, while the sides are roughly parallel (Figure 2.3).

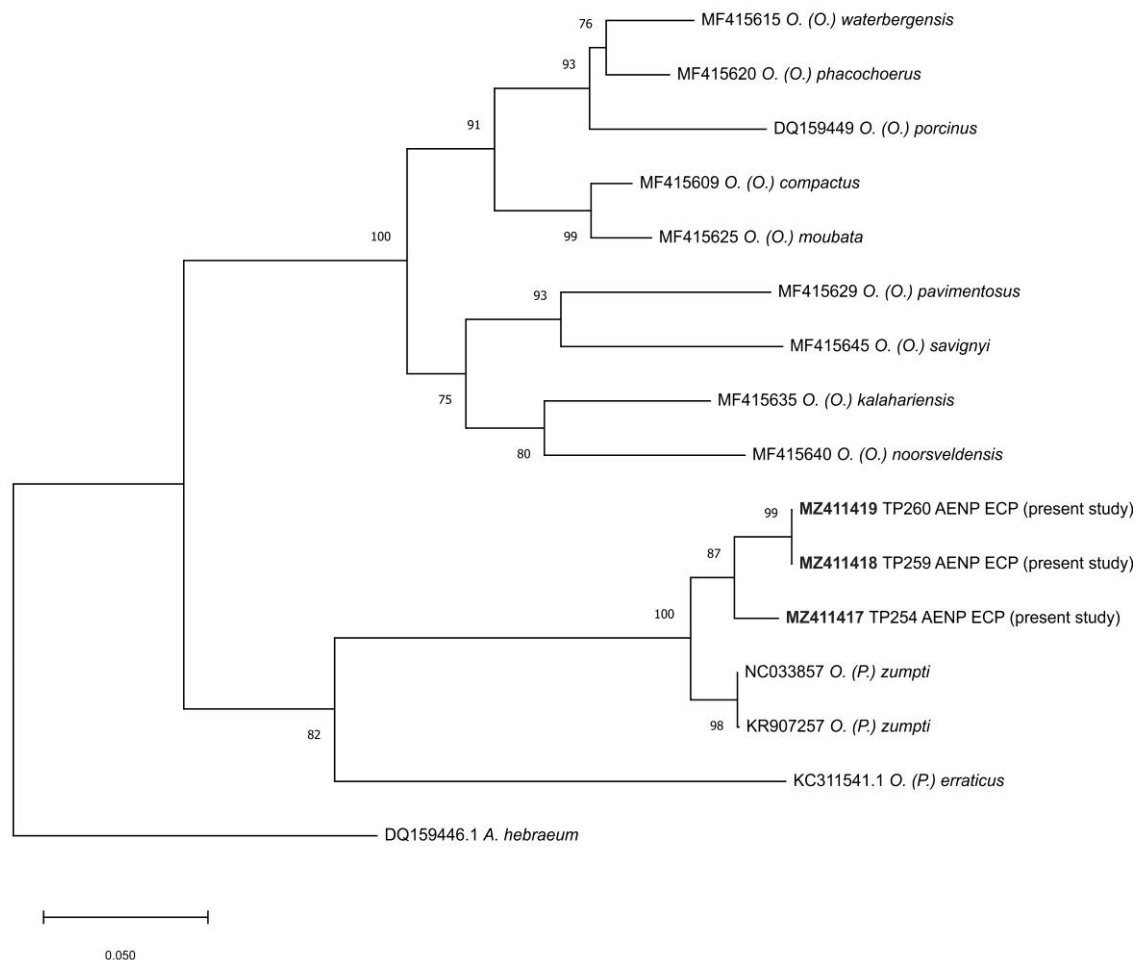


Figure 2.2: Neighbour-joining tree based on partial mitochondrial 16S rRNA gene sequence (276 nt) depicting phylogenetic relationships between the 3 *Ornithodoros (Pavlovskyella) zumpti* tick pools from the present study (GenBank accession numbers in bold) and representative *Ornithodoros* species sequences from GenBank. The corresponding gene sequence of *Amblyomma hebraeum* was included as an outlier. Percentage bootstrap support values were derived following 10,000 replications. Evolutionary analyses were conducted in MEGA X software.



Figure 2.3: Dorsal and ventral views of adult female *Ornithodoros (Pavlovskyella) zumpti* tick.

In view of the ongoing outbreaks of the ASF in the ECP it should be a priority to determine the distribution range and vector competence of *O. (P.) zumpti* ticks for ASFV, and even to investigate whether warthogs and *O. (P.) zumpti* ticks may already have become involved in circulation of the virus in that province.

2.3 Acknowledgments

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2.4 Competing interests

The authors declare that no financial or personal relationships have inappropriately influenced them in preparing this manuscript.

2.5 Ethics statement

Research ethics approval was obtained from the Animal Ethics Committee, UP, and the Research Committee of the Faculty of Veterinary Science, UP. Section 20 approval in terms of the Animal Diseases Act 35 of 1984 was obtained from the Director of Animal Health, Department of Agriculture, Land Reform and Rural Development, South Africa. Research and Materials Transfer Agreements were concluded with the Scientific Services Committee of the South African National

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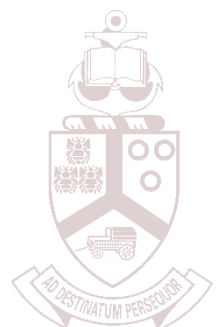
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CHAPTER 3

Extension of sylvatic circulation of African swine fever virus in extralimital warthogs in South Africa

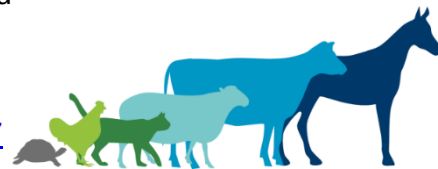


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Extension of Sylvatic Circulation of African Swine Fever Virus in Extralimital Warthogs in South Africa

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Sylvatic circulation of African swine fever virus (ASFV) in warthogs and *Ornithodoros* ticks that live in warthog burrows historically occurred in northern South Africa. Outbreaks of the disease in domestic pigs originated in this region. A controlled area was declared in the north in 1935 and regulations were implemented to prevent transfer of potentially infected suids or products to the rest of the country. However, over the past six decades, warthogs have been widely translocated to the south where the extralimital animals have flourished to become an invasive species. Since 2016, there have been outbreaks of ASF in pigs outside the controlled area that cannot be linked to transfer of infected animals or products from the north. An investigation in 2008–2012 revealed that the presence of *Ornithodoros* ticks and ASFV in warthog burrows extended marginally across the boundary of the controlled area. We found serological evidence of ASFV circulation in extralimital warthogs further south in the central part of the country.

Keywords: African swine fever virus (ASFV), sylvatic circulation, extralimital warthogs, South Africa, serosurveillance

Extension of sylvatic circulation of African swine fever virus in extralimital warthogs in South Africa

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Key words: African swine fever virus, Sylvatic circulation, Extralimital warthogs, South Africa

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3.1 Abstract

Sylvatic circulation of African swine fever virus (ASFV) in warthogs and *Ornithodoros* ticks that live in warthog burrows historically occurred in northern South Africa. Outbreaks of the disease in domestic pigs originated in this region. A controlled area was declared in the north in 1935 and regulations implemented to prevent transfer of potentially infected suids or products to the rest of the country. However, over the past six decades warthogs have been widely translocated to the south where the extralimital animals have flourished to become an invasive species. Since 2016 there have been outbreaks of ASF in pigs outside the controlled area that cannot be linked to transfer of infected animals or products from the north. An investigation in 2008-12 revealed that the presence of *Ornithodoros* ticks and ASFV in warthog burrows extended marginally across the boundary of the controlled area. We found serological evidence of ASFV circulation in extralimital warthogs further south in the central part of the country.

3.2 Introduction

African swine fever virus (ASFV) was first recognized in 1910 as the causative agent of a contagious and lethal disease of domestic pigs introduced into Kenya during the colonial era and farmed in proximity to wild suids (Montgomery, 1921). It subsequently emerged that the virus was maintained in the savannah areas of eastern and southern Africa through sylvatic circulation in the common warthog (*Phacochoerus africanus*) that does not become ill after infection and eyeless argasid ticks of the *Ornithodoros* (*Ornithodoros*) *moubata* complex that live in warthog burrows (Plowright et al., 1969; Pini, 1977; Thomson, 1985; Thomson et al., 1983). More importantly, the virus can also be maintained through uncontrolled spread in populations of domestic pigs, and this has been facilitated in recent decades by widespread increase in small-scale pig farming in Africa that frequently involves free-ranging animals and informal trading in communal and peri-urban areas (Penrith et al., 2019a; b).

In South Africa, the disease was first recognized 1926 in pigs farmed in the northernmost Limpopo Province (LP) where warthogs were present (Steyn, 1928, 1932). Outbreaks of the disease either occurred in the north of the country, or were initiated elsewhere through movement of infected pigs or pork products from the north, notably to farms in the environs of Johannesburg in Gauteng Province (GP) and from there to properties in Western Cape Province (WCP) (De Kock et al., 1940). Consequently, an ASF controlled area was declared in 1935 to include the known distribution range of warthogs at that time (Figure 3.1) (De Kock et al., 1940). Regulations were instituted to prevent movement of infected pigs or products from the controlled area, and to ensure that outbreaks were eradicated through slaughter of

infected herds plus disinfection and quarantine of premises. Carcasses of hunted or culled wild suids could be transported under veterinary permit provided that the skin, head, hooves, and internal organs were removed (De Kock et al., 1940).

The last outbreak in WCP was only eradicated in 1939. There were a few outbreaks of ASF in 1951 associated with the feeding of swill from 5 local abattoirs in GP, one of which is known to have received a consignment of infected pigs from Namibia (Neitz, 1963). In 1996, isolated outbreaks occurred on three adjoining farms in northern GP close to the boundary of the controlled area (Figure 3.2) (Penrith et al., 2013).

Thereafter, the regulatory measures approved effective until 2012 when a series of outbreaks on smallholdings in adjacent areas of GP and Mpumalanga Province (MP) were traced to initial illegal movement of infected pigs from LP followed by local spread of infection involving sale of animals at auctions (Geertsma et al., 2012; Magadla et al., 2016; Penrith et al., 2013). The virus genotype involved, XXII, had previously been detected in the district of origin of the infected pigs in LP (Janse van Rensburg et al., 2020a, b). From 2016 onwards, there have been successive series of outbreaks of ASF in domestic pigs in GP, MP, North West Province (NWP), Northern Cape Province (NCP) and Free State Province (FSP) that cannot be linked to movement of infected pigs or products from the controlled area (Figure 3.3) (DALRRD, 1993-2019; Janse van Rensburg et al., 2020a; Mulumba-Mfumu et al., 2019; WAHIS, 2005-2020). A structured serosurvey found that the outbreaks of 2016 and 2017 had been eradicated effectively, with no evidence of persistence of infection in local pig populations (Janse van Rensburg et al., 2020c).

Meanwhile, from 1963 onwards there had been widespread translocation of warthogs to the south of the country associated with the growth of an extensive wildlife ranching and conservation industry, and the extralimital animals flourished to the extent that they are regarded as an invasive species (Figure 3.3) (Penzhorn, 1971; Carruthers, 2008; Swanepoel, 2016; Swanepoel et al., 2016). An investigation conducted in 2008-12 on farms within 20 km of the boundary, revealed the presence of *Ornithodoros* ticks in warthog burrows beyond the controlled area in NWP, GP, LP and MP, with ASFV detected in one pool of ticks from MP (Magadla et al., 2016) [There is inconsistency between the text and map in Magadla et al., (2016) regarding the location of the single ASFV positive tick pool]. We were prompted to test warthog sera acquired opportunistically for evidence of sylvatic circulation of ASFV further south, and the findings are presented here.

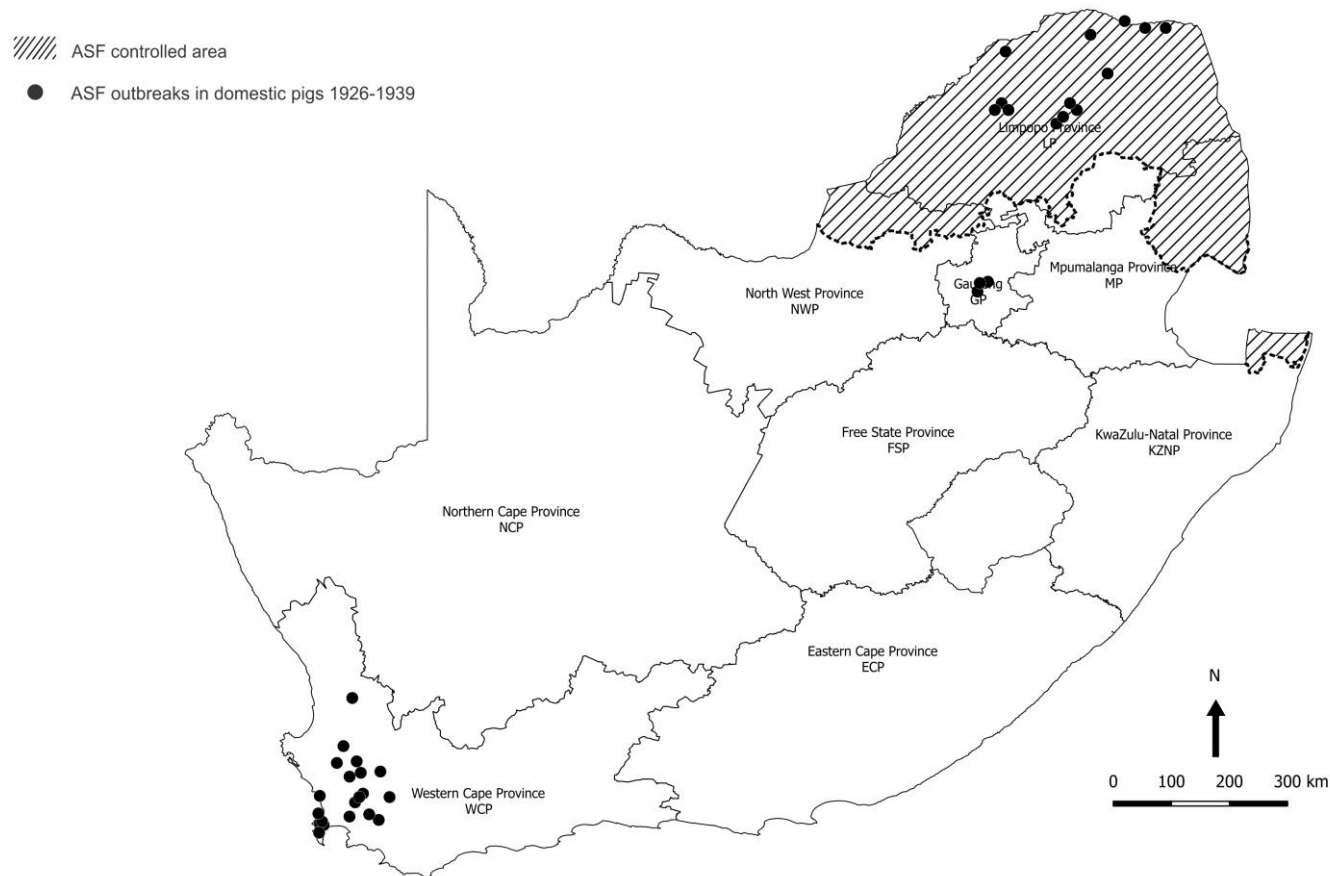


Figure 3.1: Distribution of 37 outbreaks of African swine fever in South Africa, 1926-1939, for which approximate coordinates (correct to 0.1 degree) were derived from references cited in the text. The South African Protection of Personal Information Act 4 Of 2013 precludes divulging names and accurate coordinates of private property.

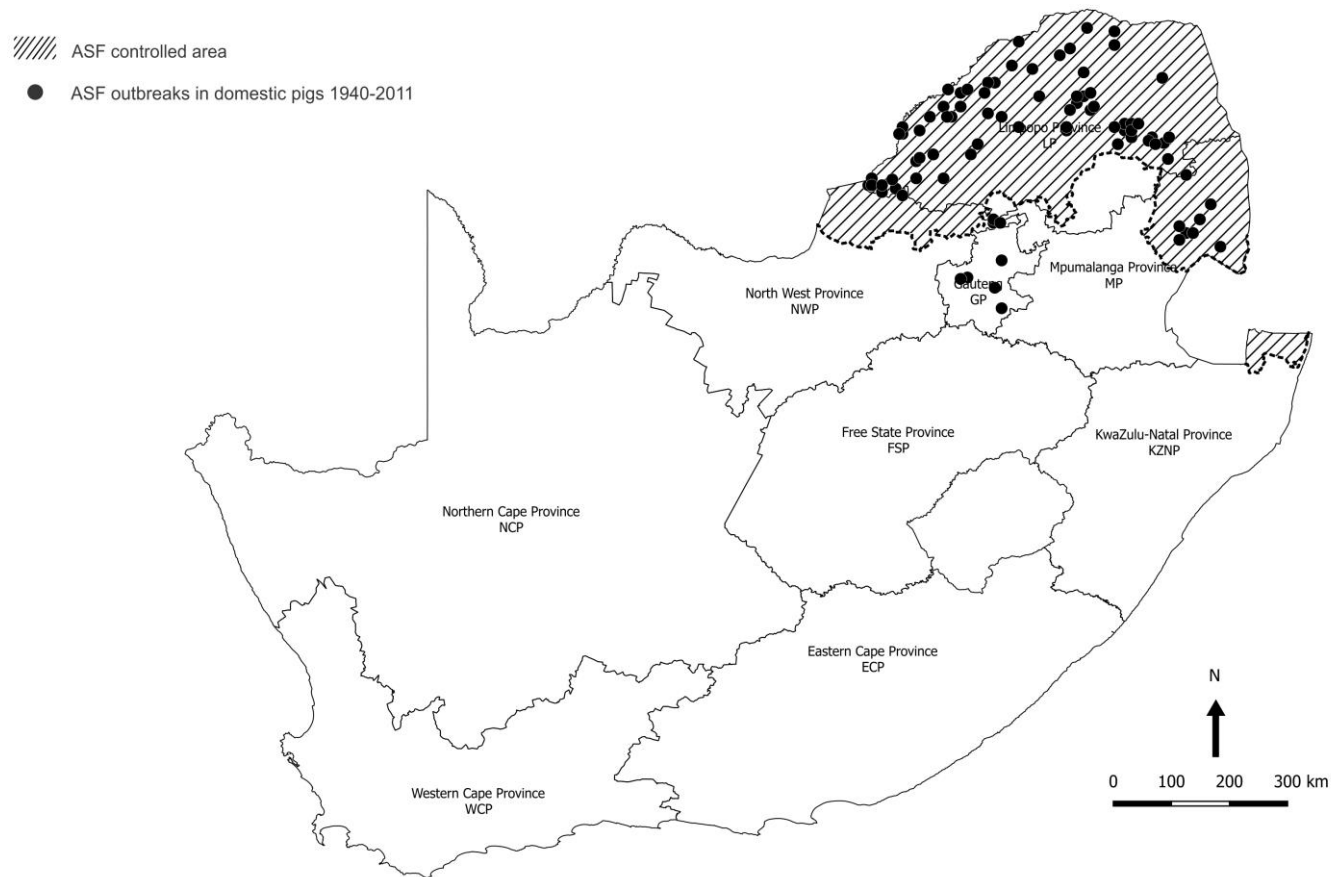


Figure 3.2: Distribution of 89 outbreaks of African swine fever in South Africa, 1940-2011, for which approximate coordinates (correct to 0.1 degree) were derived from references cited in the text.

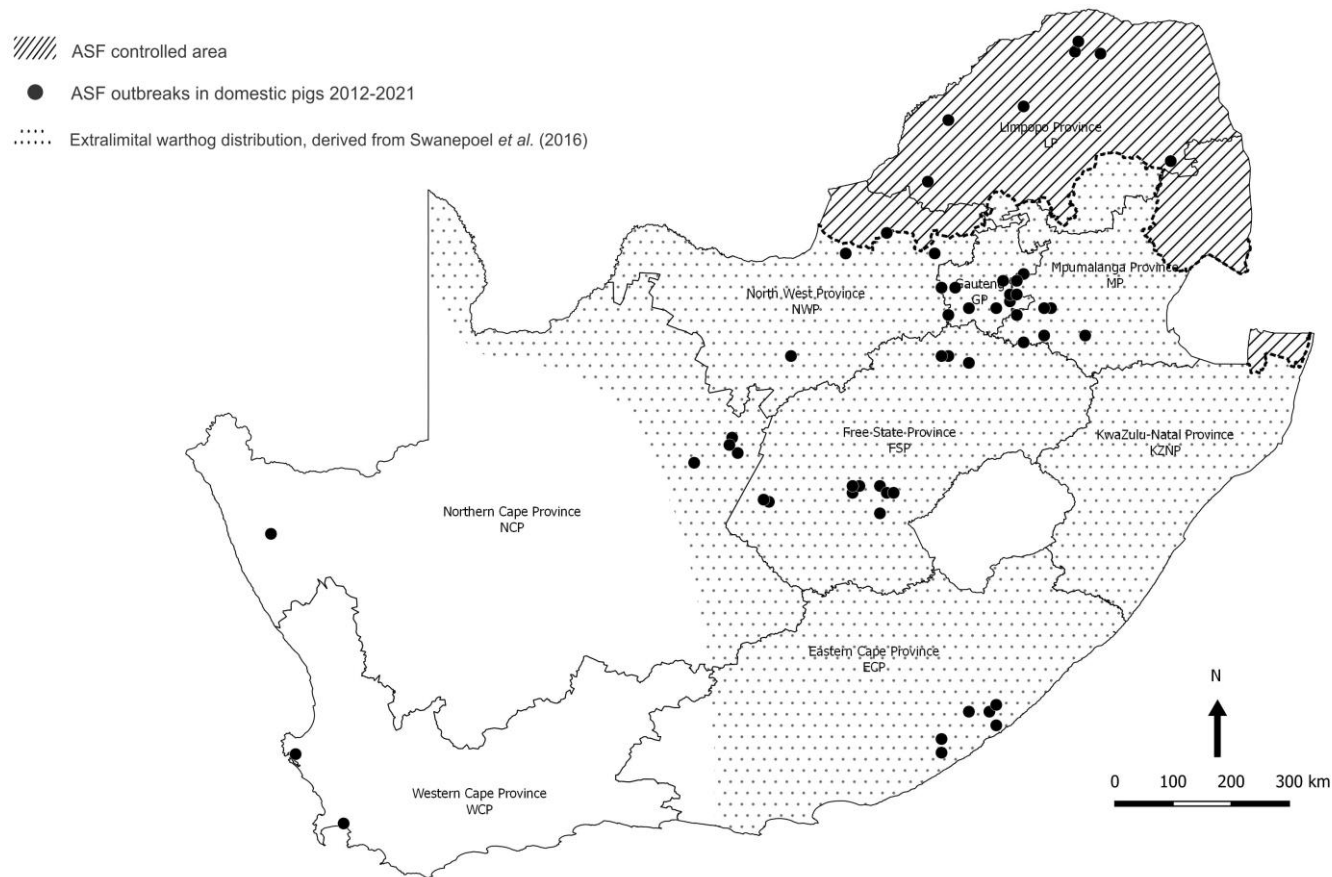


Figure 3.3: Distribution of 85 outbreaks of African swine fever in South Africa, 2012-2020, for which approximate coordinates (correct to 0.1 degree) were derived from references cited in the text. The distribution of warthogs outside the controlled area was plotted with QGIS (Penn Libraries, Philadelphia, PA) using data from Swanepoel *et al.*, (2016).

3.3 Materials and methods

3.3.1 Study sites

Baseline observations were made on serum samples obtained from warthogs in the Greater Kruger National Park (GKNP) that comprises the national park plus 20 adjoining private parks and lies within the controlled area where ASFV is known to be prevalent (Figure 3.4). For contrast, serum samples were collected in 2019 during a warthog culling operation in Addo Elephant National Park (Addo ENP) in ECP where ASF had never been recorded. Specific evidence of sylvatic circulation of ASFV beyond the controlled area was sought in the NCP by testing samples collected during a warthog culling operation in 2019 in Mokala National Park (Mokala NP), plus stored serum samples that had been collected earlier in the same park and in the provincial Rolfontein Nature Reserve (Rolfontein NR) that lies south of Mokala NP (Figure 3.4). In addition, blood samples from wild suids hunted or culled on private properties were sought through negotiation with organizations representing the wildlife industry, or collected in association with provincial veterinary officials in the vicinity of past outbreaks of ASF (Figure 3.4).

3.3.2 Samples

Altogether, 2,469 samples were collected from 546 warthogs in one provincial and three national nature reserves, including 783 duplicate tissue samples in formalin-fixative (Table 3.1).

Samples from GKNP consisted mainly of serum collected from warthogs translocated internally or culled for managerial purposes from 1999 to 2020 and stored at -80°C , but included a few blood samples and a number of lymph node and visceral organ samples collected from culled animals for an unrelated study (Roos et al., 2016) (Table 3.1).

Samples collected during the culling operations in Addo ENP and Mokala NP in 2019 comprised clotted blood for serum, whole blood collected with EDTA, mandibular, mediastinal, and mesenteric lymph nodes, plus spleen and bone marrow. Thymus and adrenal samples were collected from two fetuses encountered in Mokala NP. Samples other than blood or serum were collected mainly for a subsidiary study on distribution of ASFV in warthog tissues to be reported separately. A serial number was allocated to each warthog and the date of sampling, gender and age group estimated from size and dentition (Mason, 1984) were recorded. Access was obtained retrospectively to 23 warthog sera that had been collected during a culling operation in 2017 in Mokala NP and stored at -80°C , plus 44 warthog sera

collected in 2016 and stored at -20°C in the provincial Rolfontein NR situated approximately 100 km south of Mokala NP.

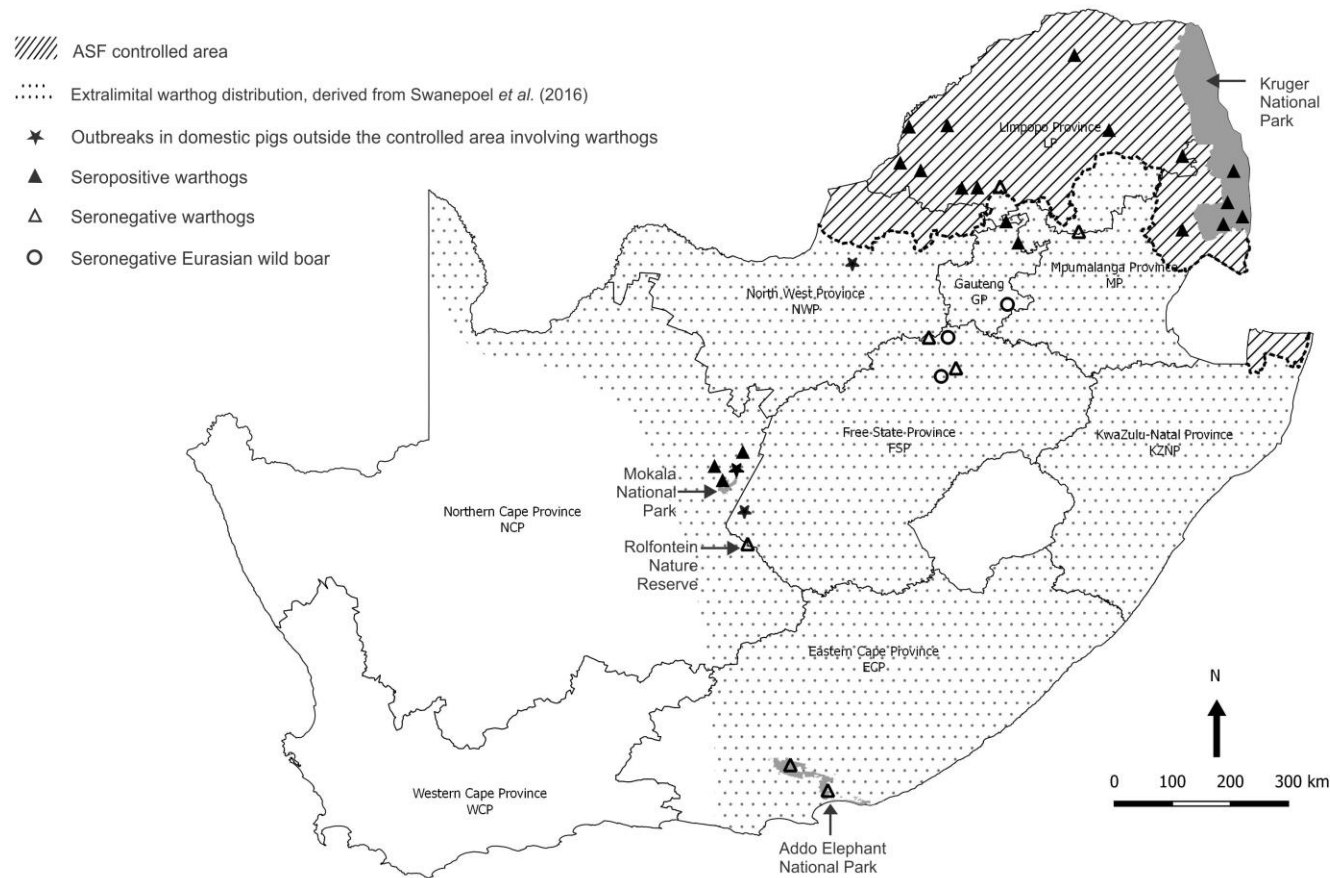


Figure 3.4: Sites where samples from warhogs and Eurasian wild boars were obtained and tested for evidence of infection with African swine fever virus, and farms outside the controlled area where African swine fever outbreaks in domestic pigs were ascribed to contact with warhogs in 2017 and 2019.

Table 3.1: Samples obtained from warthogs in four nature reserves in South Africa (abbreviations as given in the text).

Years of sampling	GKNP	Addo ENP	Mokala NP		Rolfontein NR
	1999-2020	2019	2017	2019	2016
Serum	207	116	23	77	44
EDTA blood	3	114	0	75	0
Spleen	11	36	0	82	0
Miscellaneous organs	14	0	0	0	0
Peripheral LN	95	137	0	82	0
Mediastinal LN	49	134	0	82	0
Mesenteric LN	41	14	0	81	0
Bone marrow	34	50	0	81	0
Fetal thymus	0	0	0	2	0
Fetal adrenal	0	0	0	2	0
Sub-total	454	601	23	564	44
Formalin-fixed duplicates	0	371	0	412	0
Total samples	454	972	999		44
Total warthogs sampled	241	154	107		44

Blood samples from animals hunted or culled on private properties were intended to include any wild suids that may be encountered, not just warthogs. In the event, only 48 samples were received from 19 properties (farms or small nature reserves), 10 of which were situated inside the controlled area and 9 outside (Table 3.2). The samples comprised dried blood collected on Nobuto cellulose strips (NCS) (Advantec, Tokyo, Japan) or clotted blood from warthogs and Eurasian wild boars submitted by landowners and hunters from properties 1 to 17, plus clotted blood, mediastinal lymph node and spleen samples from 4 warthogs collected in association with provincial veterinary officials during investigations on properties 18 and 19 in the vicinity of past ASF outbreaks in NCP (Table 3.2).

3.3.3 Detection of antibody to ASFV

Tests for antibody to ASFV protein 72 (VP72) in serum samples were performed with INgezim PPA Compac R. 11.PPA.K3 blocking enzyme-linked immunosorbent assay (ELISA) kits (Eurofins Technologies Ingenasa, Madrid, Spain) used according to the manufacturer's instructions. Dried blood samples on NCS were eluted with kit buffer before testing (Randriamparany et al., 2014).

Table 3.2: Dried blood samples on Nobuto cellulose strips (Ncs) and clotted blood samples from warthogs or Eurasian wild boars received from hunters or landowners from 10 properties inside and 9 outside the African swine fever controlled area in South Africa during 2019 and 2020 (abbreviations as given in the text).

Property	Municipal area	Province	Species	Sample	No. of samples	ASF ELISA positive
Properties inside the ASF controlled area						
1	Makhado	LP	Warthog	Ncs	3	3
2	Lephalale	LP	Warthog	Ncs	4	4
3	Lephalale	LP	Warthog	Ncs	3	3
4	Thabazimbi	LP	Warthog	Ncs	4	4
5	Thabazimbi	LP	Warthog	Ncs	2	2
6	Thabazimbi	LP	Warthog	Ncs	3	3
7	Modimolle	LP	Warthog	Ncs	1	0
8	Bela Bela	LP	Warthog	Ncs	1	1
9	Bela Bela	LP	Warthog	Ncs	4	4
10	Mbombela	MP	Warthog	Ncs	4	2
Properties outside the ASF controlled area						
11	Bela Bela	LP	Warthog	Ncs	2	2
12	Elias Motsoaledi	LP	Warthog	Ncs	1	0
13	Tlokwe	NWP	Eu wild boar	Blood	1	0
13	Tlokwe	NWP	Warthog	Blood	1	0
14	Ekurhuleni	GP	Eu wild boar	Blood	1	0
15	Tshwane	GP	Warthog	Ncs	2	2
16	Ngwathe	FSP	Warthog	Ncs	2	0
17	Ngwathe	FSP	Eu wild boar	Ncs	1	0
18	Dikgatlong	NCP	Warthog	Blood*	2	2
19	Dikgatlong	NCP	Warthog	Blood*	2	2
					44	34

**Additionally, mediastinal lymph node and spleen samples were obtained from the same animals.*

3.3.4 Nucleic acid extraction, ASFV DNA detection and p72 genotyping

Approximately 10% (w/v) suspensions of warthog tissues were prepared by homogenizing samples in phosphate-buffered saline, pH7.2 (PBS). Automated nucleic acid extraction was

performed with IndiMag Pathogen kits (Indical Bioscience, Leipzig, Germany) using slight modifications to the manufacturer's instructions. Briefly, 200µL of tissue homogenate supernatant was added to 300µL ATL buffer (Qiagen, Dusseldorf, Germany) and 40µL of proteinase K (Qiagen) and incubated at 56°C for 2 hours before 200µL of the lysed sample was added to 200µL AL buffer (Qiagen) and incubated at 70°C for 10min. For whole blood samples, 200µL was added directly to 200µL AL buffer and mixed well. For all samples, 200µL of the AL lysate was added to the IndiMag buffer for extraction. Each extraction included known ASFV positive controls. Eluates were stored at -80°C until further use.

Eluates were tested for ASFV nucleic acid using the real-time quantitative PCR (qPCR) assay of Zsak et al. (2005) with modifications (Sunwoo et al., 2019). Briefly, 5µL DNA was amplified in 20µL reactions using 20 pmol of the published primers and 7 pmol of probe in Perfecta Fastmix II (Quanta Biosciences, Beverly, MA). Positive and no template controls (NTC) were included for each PCR run. Samples with Cq mean values ≤ 38 (selected as the cut-off value based on the analytical sensitivity limits of the qPCR assay) were considered positive.

To confirm that ASFV was identified in warthog tissues from Mokala NP, nucleic acid from the qPCR positive spleen sample with lowest Cq mean value, was amplified using primers *p72-D* and *p72-U* and the cycling conditions of Bastos et al. (2003). An appropriately-sized band of amplification product was excised from the agarose electrophoresis gel, purified and subjected to Sanger nucleotide sequencing. The sequence was viewed, and maximum likelihood phylogenetic comparisons made with representative sequences of the 24 known ASFV *p72* genotypes (Quembo et al., 2018) using MEGA X software (Kumar et al., 2018).

3.4 Results

The results obtained in the ELISA for antibody to ASFV in warthog sera from the four nature reserves are presented in figures 3.4 and 3.5. As expected, a high prevalence of antibody was found in the sera of warthogs from GKNP, 97.1% (201/207), with doubtful reactions recorded in a further two juveniles, while no antibody activity was detected in sera from Addo ENP in ECP where no ASF outbreaks had ever been recorded. In contrast, a high prevalence of antibody was detected in sera collected during the 2019 culling operation in Mokala NP, 98.7% (76/77), some 400 km south of the controlled area, and also in the sera collected there in 2017, 91.3% (21/23), while no antibody was detected in the 44 samples collected in Rolfontein NR in 2016.

A high prevalence of antibody, 26/29 (89.6%), was detected in dried warthog blood samples obtained from 9/10 private properties within the ASF controlled area (Table 3.2; Figure 3.4).

Outside the controlled area antibody was detected on only 4/9 properties. These included positive reactions in 4 warthog blood samples from properties 11 and 15 (a small nature reserve), that lie close to each other and to the 3 farms where ASF outbreaks occurred in 1996 within 40 km south of the boundary of the controlled area near Pretoria in northern GP, plus 4 warthog sera from properties 18 and 19 that are situated about 100 km north and west of Mokala NP in the vicinity of past outbreaks of ASF in pigs in NCP. The remaining samples tested negative despite the fact that two sera from properties 13 and 17 in NWP and FSP came from Eurasian wild boars shot as free-ranging animals in proximity to free-ranging warthogs. In the course of the investigations histories were obtained to confirm that fresh warthog offal had been fed to pigs on one farm where an outbreak of ASF had occurred in NCP in 2017, and that free-roaming pigs had been in contact with warthogs, including carcasses, on a farm where an outbreak occurred in the adjacent eastern* FSP in 2017, with both locations being over 100 km distant from Mokala NP (Table 3.2; Figure 3.4) (WAHIS, 2005-2020). In addition, an outbreak of ASF in 2019 involved both pigs and Eurasian wild boars that had escaped their pens on a farm where warthogs were present 10 km south of the controlled area in NWP (Table 3.2; Figure 3.4) (WAHIS, 2005-2020).

The ELISA is not accredited for detection of ASF antibody in warthog sera and in order to confirm the presumptive evidence for presence of the virus in Mokala NP, all 75 warthog EDTA blood samples and the 412 other tissue samples collected in Mokala NP in 2019 were tested for ASFV nucleic acid by qPCR (Zsak et al., 2005; Sunwoo et al., 2019). All EDTA blood samples tested negative but 13 of the other samples from Mokala NP, including mandibular, mediastinal and mesenteric lymph nodes plus spleen tested positive. The sample with the lowest C_q mean value, spleen from warthog Mokala NP 77, was selected for PCR with the Bastos et al. (2003) *p72* primers and the product determined to belong to the *p72* genotype I of ASFV as had been identified in the disease outbreaks outside the controlled area in 2016 and subsequently in the same districts (Janse van Rensburg et al., 2020a). The more recent outbreaks of ASF that occurred in ECP and WCP in 2020 and 2021 subsequent to the present sampling (Figure 3.3), were associated with genotype II virus, also detected in an outbreak in NWP in 2019 on the boundary of the controlled area, but otherwise associated with Mozambique, Madagascar, Mauritius, Tanzania, Zambia and Zimbabwe (Bastos et al., 2003; Bastos et al., 2004; Penrith et al., 2019a; b; DALRRD, 2020; 2021; WAHIS, 2005-2020).

3.5 Discussion

Bushpigs (*Potamochoerus larvatus*) are the only indigenous wild suids present in South Africa apart from warthogs (Venter et al., 2016). They are widely distributed in wooded areas and

sleep in lairs. Consequently, they do not have the same exposure to *Ornithodoros* ticks in burrows as warthogs. Bushpigs do not become ill following experimental inoculation with ASFV but develop viremic infection and can be transiently contagious for domestic pigs (Anderson et al., 1998; Oura et al., 1998; Salguero, 2020; Thomson et al., 1980). Natural infection of bushpigs with ASFV, presumed to involve environmental contamination, has been reported in East Africa with some evidence of transmission of virus to domestic pigs (De Tray, 1963; Kukiela et al., 2016; Okoth et al., 2013; Ståhl et al., 2014).

In South Africa, bushpigs were stated to be less commonly infected with ASFV than warthogs and have never been implicated in outbreaks of the disease in domestic pigs, although on occasion warthogs and Eurasian wild boars have been misidentified as bushpigs (Mansvelt, 1963; Janse van Rensburg et al., 2020b). They are less frequently hunted than warthogs and no samples from bushpigs were received for testing during the present study.

Feral domestic pigs and Eurasian wild boars, *Sus scrofa ferus*, are exotic to sub-Saharan Africa. They are susceptible to the disease and can be involved in spread of infection by contagion. A recently discovered mechanism for maintenance of ASFV involves survival of infectivity in carcasses in the cold climate of northern Europe and scavenging by wild boars (Penrith et al., 2019b, Chenais et al., 2018).

Eurasian wild boars were introduced into South Africa to control pine tree moths in WCP where residual populations still exist, but apparently they did not adapt well to conditions in that region (Botha, 1989). Attempts were made to farm with Eurasian wild boars and cross-bred domestic pigs inside the controlled area in LP and MP, and to utilize them for hunting, but the animals succumbed to ASFV (Penrith, 2014; DALRRD, 1993-2019; Janse van Rensburg et al., 2020b; WAHIS, 2005-2020). As mentioned in results, an outbreak of ASFV involving domestic pigs and Eurasian wild boars occurred on a farm where warthogs were present in NWP in 2019, and 3 blood samples were received from wild boars in NWP, GP and FSP for the present project (Table 3.2; Figure 3.4). Since Eurasian wild boars usually succumb to ASFV infection it was to be expected that the 3 blood samples tested negative for antibody to the virus, but the presence of these animals in NWP, GP and FSP suggests that there is need to monitor the distribution and growth of populations in South Africa, particularly since the species has proved to be a highly invasive elsewhere.

The present findings confirm that there is circulation of ASFV in warthogs beyond the controlled area in South Africa (Table 3.2; Figure 3.4; 3.5), but the full extent of spread of infection and the mechanisms involved remain largely undetermined. Unbeknown to the present authors, aliquots of 91 of the same GKNP sera were included in an unrelated survey

with similar ASFV findings (Neiffer et al., 2021), but this is immaterial since the samples were used in the current study mainly to confirm the validity of the antibody test for warthog sera. Samples from extralimital warthogs were of central interest.

Warthog piglets are infected by ticks while confined to burrows during early life and develop intense viremia that in turn facilitates infection of ticks (Thomson, 1985). Low intensity viremia and infection of lymphatic tissues may persist for months but warthogs are not contagious for domestic pigs or each other (Montgomery, 1921; Thomson et al., 1980). There is controversy about the infectivity of warthog offal for domestic pigs, but it has recently been confirmed that low doses of virus are infective for pigs by mouth (Penrith et al., 2019b; Niederwerder et al., 2019). The majority of outbreaks of ASF recorded within the controlled area in South Africa involved known contact between domestic pigs and live warthogs, carcasses, or offal (Janse van Rensburg et al., 2020b).

Transmission of ASFV by ticks was discovered in Spain after introduction of the virus into Europe and involved domestic pigs and *Ornithodoros (Pavlovskyella) erraticus* ticks (Sanchez-Botija, 1963). Circulation of ASFV in domestic pigs and *O. (O.) moubata* complex ticks living in crevices of poorly constructed animal shelters was documented in Malawi and probably occurs elsewhere in Africa, but has not been reported in South Africa (Haresnape and Mamu, 1986; Penrith et al., 2019b). *Ornithodoros* ticks generally engorge blood meals in minutes to hours and detach while animals are at rest but can be conveyed passively on their hosts between burrows or to the environs of domestic pigs to transmit infection (Plowright et al., 1994; Horak et al., 1983, Horak et al., 1989, Boomke et al., 1991). Transport of *Ornithodoros* ticks on live warthogs or carcasses to the environs of piggeries has been observed in the controlled area (Janse van Rensburg et al., 2020b).

The original warthog translocations in South Africa were undertaken by officials of national and provincial parks in order to replace the Cape warthog (*P. aethiopicus aethiopicus*), formerly present in the south of the country but extinct after the rinderpest epidemic of 1896 (Grubb, 1993; Vercammen and Mason, 1993). Warthogs were sourced from the Hluhluwe-iMfolozi Game Reserve in KwaZulu-Natal Province considered to be free of *Ornithodoros* ticks and ASFV at the time (Penzhorn, 1971; Penrith et al., 2019b). Translocations were made to the ECP in 1976-77 and from there to Rolfontein NR in NCP in 1984. From 1991 onwards, Rolfontein NR served as the source for transfers to other reserves in NCP (Somers and Penzhorn, 1992; Swanepoel, 2016). The lack of antibody to ASFV in warthog sera collected in Rolfontein NR in 2016 and Addo ENP in ECP in 2019 (Figure 3.5), implies that the virus was not disseminated during the original warthog translocations.

When Mokala NP was established in 2007 many animals were transferred from the former Vaalbos National Park in NCP, but warthogs were not included since they were already present on the farms incorporated into the new park (Mr J. de Klerk, Manager, Mokala NP, personal communication, 2019). Hence, the high prevalence of antibody found in Mokala NP in 2017 and 2019 implies that the virus is endemic in the region, not just in the park. This is consistent with the detection of seropositive warthogs on farms 18 and 19 (Table 3.2) and the histories of contact of domestic pigs with warthog tissues in the outbreaks of ASF recorded on farms in NCP and adjacent FSP in 2017 (Figure 3.4).

Major nature reserves have not been implicated in triggering outbreaks of ASF in domestic pigs in South Africa, but the large populations of warthogs present on private land appear to play a direct role. To what extent multiple unrecorded translocations of warthogs that accompanied the burgeoning of game ranches and private nature reserves (Maciejewski and Kerley, 2014; Swanepoel, 2016) promoted dispersal of ticks and ASFV is less important than the potential threat posed by continued expansion of extralimital warthog populations and possible utilization without due regard to biosafety measures. The threat is greater for small-scale pig farmers who exercise little control on potential introduction of infection (Penrith et al., 2019a; Fasina et al., 2015; Etter et al., 2019) than it is for commercial producers that apply strict compartmentalization procedures.

The taxonomy of Afrotropical *Ornithodoros* ticks was recently reviewed with description of new species and type localities, but little is known about ASFV vector competence and distribution ranges for some species (Bakkes et al., 2018). Observations on the occurrence of *Ornithodoros* tick species and their ASFV infection status in relation to a selection of the current study sites will be presented separately.

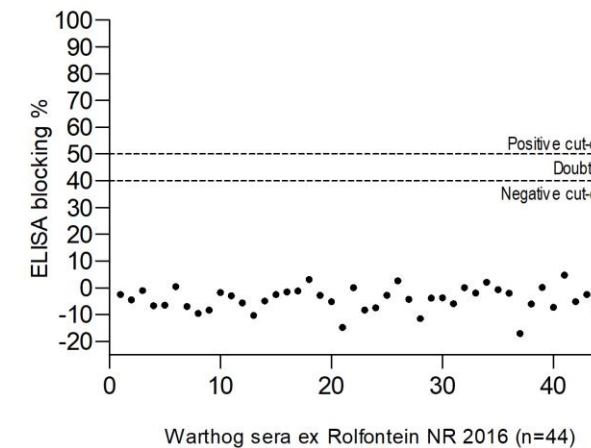
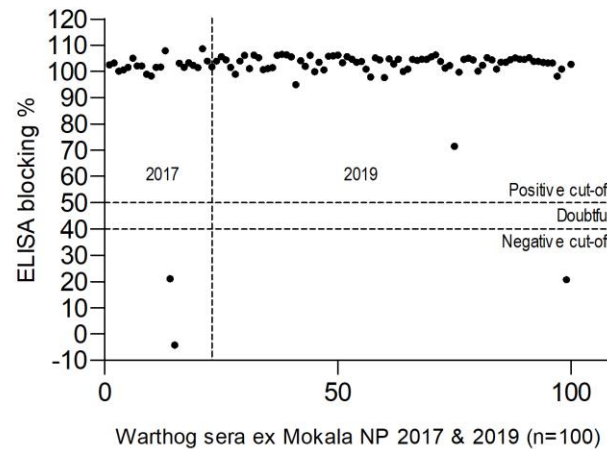
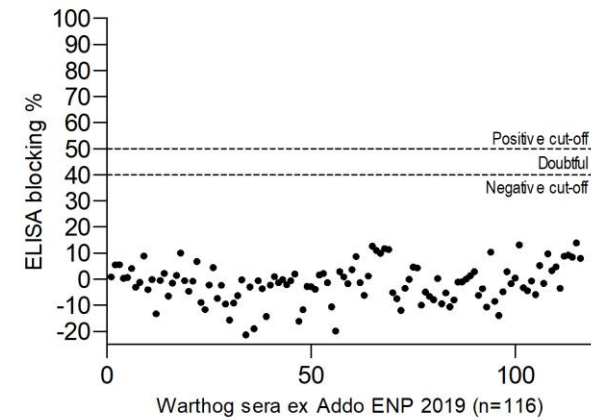
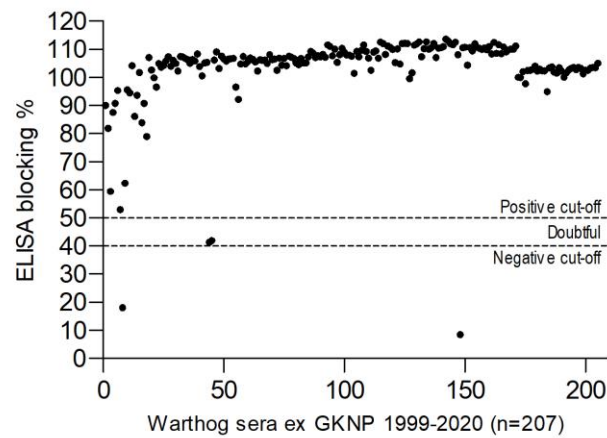


Figure 3.5: Results of blocking enzyme-linked immunosorbent assays (ELISA) for antibody to African swine fever virus performed on warthog sera obtained from four nature reserves in South Africa (abbreviations as given in the text).

3.6 Acknowledgments

We thank the South African Hunters and Game Conservation Association for arranging submission by their members of blood samples from hunted warthogs and Eurasian wild boars. We thank Dr Angela Brüns of Veterinary Wildlife Services, South African National Parks, Kimberley, for samples collected during the 2017 culling operation in Mokala NP, and the ASF research team members of the Agricultural Research Council-Onderstepoort Veterinary Research, Transboundary Animal Diseases Laboratory (TADL) for technical assistance and guidance. The project was supported by a research contract from Kansas State University and a grant awarded by the South African Agricultural Sector Education and Training Authority (AgriSETA) to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria (UP). We acknowledge the support of NBAF Transition funds from the State of Kansas, the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health under award number P20GM130448, and the Department of Homeland Security Center of Excellence for Emerging and Zoonotic Animal Diseases under grant number HSHQDC 16-A-B0006 to JAR.

3.7 Competing interests

The authors declare that no financial or personal relationships have inappropriately influenced them in preparing this manuscript.

3.8 Ethics statement

Research ethics approval was obtained from the Animal Ethics Committee, UP, and the Research Committee of the Faculty of Veterinary Science, UP. Section 20 approval in terms of the Animal Diseases Act 35 of 1984 was obtained from the Director of Animal Health, Department of Agriculture, Land Reform and Rural Development, South Africa. Research and Materials Transfer Agreements were concluded with the Scientific Services Committee and Veterinary Wildlife Services of the South African National Parks Board, and written informed consent was obtained from the owners of other properties from which samples were received. Samples were transported in appropriate biosafety packaging under permit from relevant State Veterinarians and laboratory procedures were performed under Animal BSL3 conditions in TADL.

* Erratum: *eastern FSP should read western FSP*, journal being notified.

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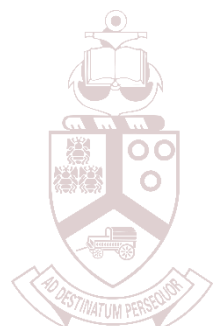
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CHAPTER 4

Detection of African swine fever virus in *Ornithodoros* tick species associated with indigenous and extralimital warthog populations in South Africa



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Article

Detection of African Swine Fever Virus in *Ornithodoros* Tick Species Associated with Indigenous and Extralimital Warthog Populations in South Africa

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Detection of African swine fever virus in *Ornithodoros* tick species associated with indigenous and extralimital warthog populations in South Africa

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Keywords: African swine fever virus (ASFV), sylvatic circulation, *Ornithodoros* ticks, South Africa

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4.1 Abstract

We investigated the possibility that sylvatic circulation of African swine fever virus (ASFV) in warthogs and *Ornithodoros* ticks had extended beyond the historically affected northern part of South Africa that was declared a controlled area in 1935 to prevent spread of infection to the rest of the country. We recently reported finding antibody to the virus in extralimital warthogs in the south of the country, and now describe detection of infected ticks outside the controlled area. A total of 5,078 ticks was collected at 45 locations in 7/9 provinces during 2019-2021 and assayed as 711 pools for virus content by qPCR, while 221 pools were also analysed for tick phylogenetics. Viral nucleic acid was detected in 50 tick pools representing all four members of the *Ornithodoros* (*Ornithodoros*) *moubata* complex known to occur in South Africa: *O. (O.) waterbergensis* and *O. (O.) phacochoerus* species yielded ASFV genotypes XX, XXI, XXII at 4 locations and *O. (O.) moubata* yielded ASFV genotype I at two locations inside the controlled area. Outside the controlled area, *O. (O.) moubata* and *O. (O.) compactus* ticks yielded ASFV genotype I at 7 locations, while genotype III ASFV was identified in *O. (O.) compactus* ticks at a single location. Two of the three species of the *O. (O.) savignyi* complex ticks known to be present in the country, *O. (O.) kalahariensis* Bakkes et al., 2018 and *O. (O.) noorsveldensis* Bakkes et al., 2018, were collected at single locations and found negative for virus. The only member of the *Pavlovskyella* subgenus of *Ornithodoros* ticks known to occur in South Africa, *O. (P.) zumpti*, was collected from warthog burrows for the first time, in Addo National Park in Eastern Cape Province where ASFV had never been recorded, and it tested negative for the viral nucleic acid. While it is confirmed that there is sylvatic circulation of ASFV outside the controlled area in South Africa, there is need for more extensive surveillance and for vector competence studies with various species of *Ornithodoros* ticks.

4.2 Introduction

African swine fever virus (ASFV) causes a contagious and lethal disease of domestic pigs. In the savannah areas of eastern and southern Africa, the virus is maintained in sylvatic circulation between warthogs that develop benign viremic infection and eyeless ticks of the *Ornithodoros* (*Ornithodoros*) *moubata* complex that live in warthog burrows [1-3]. In South Africa, the disease in domestic pigs was first recognized in the north of the country, where the common warthog (*Phacochoerus africanus*) was prevalent [4]. Consequently, a controlled area was declared in the north in 1935 and regulations were implemented to prevent transfer of infected suids or products to the rest of the country [5].

The regulations initially proved effective, however, from 2016 there were outbreaks of the disease in domestic pigs in the south that could not be linked to recent transfer of infected animals or materials from the controlled area. The genotype of ASFV involved, genotype I, had been isolated decades earlier from outbreaks of disease in pigs in South Africa, and from ticks inside the controlled area. The same genotype had long been associated with the disease in domestic pigs in countries of western Africa from where it is believed to have been accidentally introduced into Europe in 1957 and 1960 [6-14]. In South Africa, since 1963, there has been widespread translocation of warthogs to game farms and nature reserves in the south of the country, originally from a source considered to be free of ticks and virus, but in 2008-2012 *Ornithodoros* ticks were found in warthog burrows on farms approximately 20 km south of the controlled area, including ASFV-infected ticks in one instance [15-19].

Although the most recent outbreaks of the disease outside the controlled area were of undetermined origin, there were indications that contact with warthogs was involved in at least three instances in the North West, Northern Cape and Free State Provinces (Figure 4.1) [6-9,11,12]. Hence, we sought evidence of the occurrence of sylvatic circulation of ASFV outside the controlled area and found antibody to the virus in opportunistically acquired serum samples from extralimital warthogs [20]. We now report detection of ASFV nucleic acid in *Ornithodoros* ticks collected from warthog burrows both within and beyond the controlled area in South Africa.

The taxonomy of the Afrotropical *Ornithodoros* ticks was recently revised with description of new species [21], and the present observations incidentally extend the information available on the distribution ranges, host associations and potential roles of certain tick species as vectors of ASFV. Moreover, tests for antibody to ASFV were performed on a limited number of blood and serum samples from tortoises and hyaenas to determine whether they are exposed to infection as alternative hosts for *Ornithodoros* ticks.

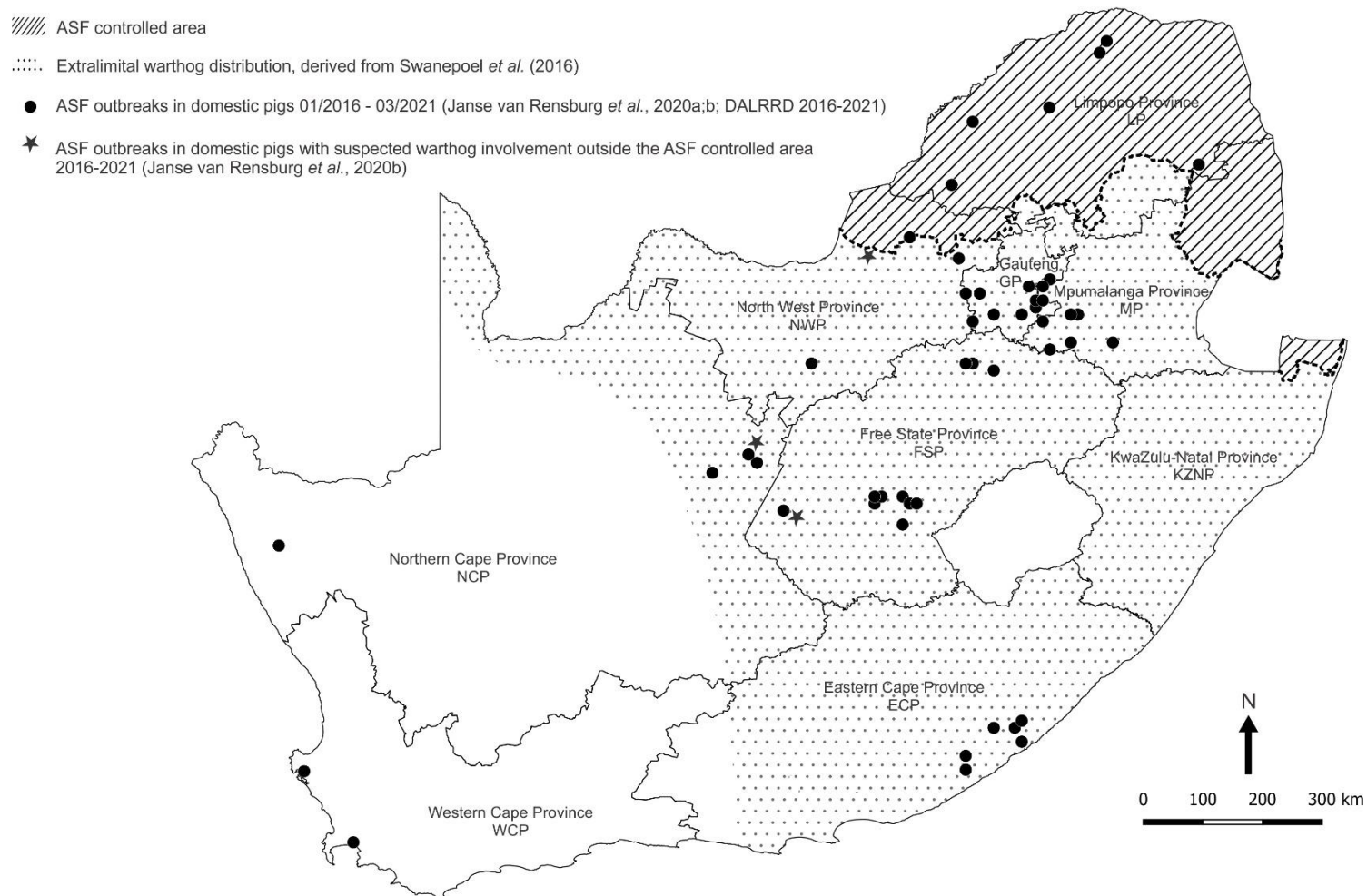


Figure 4.1: Spatial distribution of outbreaks of African swine fever in domestic pigs in relation to the ASF controlled area and the extralimital distribution of warthogs in South Africa, 2016-2021. Approximate coordinates (correct to 0.1 degree) were derived from references cited in the text. The South African Protection of Personal Information Act 4 of 2013 precludes divulging names and accurate coordinates of private property.

Table 4.1: Summary of locations where *Ornithodoros* ticks were collected, numbers of ticks collected and pools tested and found positive for African swine fever virus nucleic acid by qPCR, genotypes of virus identified, numbers of tick pools subjected to 16S rRNA sequencing, and species of ticks identified.

Location	Province ¹	Location type	QDGC ²	Collection sites	Ticks collected	Pools tested	ASFV qPCR positive	ASFV p72 genotype	16S rRNA sequences	Tick species
Locations inside ASF controlled area										
LP01	LP	Farm	E029S022CB	1	218	7			6	<i>O. (O.) waterbergensis</i>
LP02	LP	Farm	E027S024CA	1	2	1				<i>O. (O.) waterbergensis</i>
LP03	LP	Farm	E026S024DB	3	160	27	1	XXII	2	<i>O. (O.) waterbergensis</i>
GKNP01	LP	Nature Reserve	E031S024AC	1	82	12	1	XX	12	<i>O. (O.) phacochoerus</i>
GKNP02	LP	Nature Reserve	E031S022CA	1	316	46			2	<i>O. (O.) phacochoerus</i>
GKNP03	LP	Nature Reserve	E031S022CA	1	26	4			2	<i>O. (O.) phacochoerus</i>
GKNP04	LP	Nature Reserve	E031S023CC	1	192	64			2	<i>O. (O.) phacochoerus</i>
GKNP05	LP	Nature Reserve	E031S023DC	1	115	15			2	<i>O. (O.) phacochoerus</i>
GKNP06	LP	Nature Reserve	E031S023CD	1	60	11			1	<i>O. (O.) phacochoerus</i>
GKNP07	MP	Nature Reserve	E031S024BA	1	104	31	2	XXI	31	<i>O. (O.) phacochoerus</i>
GKNP08	MP	Nature Reserve	E031S024BA	1	150	26			1	<i>O. (O.) phacochoerus</i>
GKNP09	MP	Nature Reserve	E031S024BC	1	58	10			1	<i>O. (O.) phacochoerus</i>
GKNP10	MP	Nature Reserve	E031S024BC	1	23	8			1	<i>O. (O.) phacochoerus</i>
GKNP11	MP	Nature Reserve	E031S024DB	1	129	36			1	<i>O. (O.) phacochoerus</i>
GKNP12	MP	Nature Reserve	E031S024DD	1	84	8			1	<i>O. (O.) phacochoerus</i>
GKNP13	MP	Nature Reserve	E031S024DB	1	157	15			1	<i>O. (O.) phacochoerus</i>
GKNP14	MP	Nature Reserve	E031S025BA	4	558	25			2	<i>O. (O.) phacochoerus</i>
GKNP15	MP	Nature Reserve	E031S025BA	1	56	10			2	<i>O. (O.) phacochoerus</i>
GKNP16	MP	Nature Reserve	E031S025BC	1	219	57			1	<i>O. (O.) phacochoerus</i>
GKNP17	MP	Nature Reserve	E031S025BD	1	120	14			1	<i>O. (O.) phacochoerus</i>
GKNP18	MP	Nature Reserve	E031S025BD	4	254	14	4	XX, XXI	4	<i>O. (O.) phacochoerus</i>
NWP01	NWP	Farm	E026S024CD	5	148	25	3	la	3	<i>O. (O.) moubata</i>
NWP02	NWP	Farm	E026S025AA	3	12	1			1	<i>O. (O.) moubata</i>
NWP03	NWP	Farm	E026S025AA	1	136	10	2	lb	3	<i>O. (O.) moubata</i>

Locations outside ASF controlled area

NWP04	NWP	Nature Reserve	E026S025CA	1	7	2	1	lb	1	<i>O. (O.) moubata</i>
NWP05	NWP	Farm	E026S025CA	2	227	37	6	lb	5	<i>O. (O.) moubata</i>
MP01	MP	Nature Reserve	E029S025CA	10	0					
GP01	GP	Nature Reserve	E028S025BC	1	8	5			4	<i>O. (O.) moubata</i>
GP02	GP	Nature Reserve	E028S025AD	3	92	6			2	<i>O. (O.) moubata</i>
GP03	GP	Farm	E028S025CB	1	1	1			1	<i>O. (O.) moubata</i>
GP04	GP	Nature Reserve	E028S025BA	1	10	5			5	<i>O. (O.) moubata</i>
GP05	GP	Nature Reserve	E028S025BC	1	10	4			2	<i>O. (O.) moubata</i>
GP06-GP07	GP	Farms	E027S026CB	2	0					
GP08-GP11	GP	Farms	E027S026CA	4	0					
GP12-GP13	GP	Farms	E028S025DA	2	0					
GP14-GP18	GP	Farms	E028S025CB	5	0					
MNP01	NCP	Nature Reserve	E024S029AB	6	287	54	4	la, lc	53	<i>O. (O.) compactus</i>
NCP01	NCP	Farm	E024S028BC	3	316	51	19	la, lb, lc ³	19	<i>O. (O.) compactus</i>
NCP02	NCP	Farm	E024S028AD	2	86	11	3	la,b	4	<i>O. (O.) compactus</i>
NCP03	NCP	Nature Reserve	E024S028BC	6	65	9			1	<i>O. (O.) compactus</i>
NCP04 ⁴	NCP	Nature Reserve	E024S028CB	1	32	3	1	la	3	<i>O. (O.) compactus</i>
NCP04 ⁴	NCP	Nature Reserve	E024S028CB	1	27	1			1	<i>O. (O.) kalahariensis</i>
NCP05	NCP	Farm	E023S028DD	1	53	2			1	<i>O. (O.) compactus</i>
NCP06	NCP	Farm	E022S028CC	3	31	3	1	I	1	<i>O. (O.) compactus</i>
FSP01	FSP	Nature Reserve	E024S028DD	1	2	1			1	<i>O. (O.) compactus</i>
FSP02	FSP	Farm	E025S029AC	3	97	7	1	lc	3	<i>O. (O.) compactus</i>
FSP03	FSP	Farm	E024S029BD	1	8	1	1	III	1	<i>O. (O.) compactus</i>
AENP01	ECP	Nature Reserve	E025S033BD	1	7	1			1	<i>O. (P.) zumpti</i>
AENP02	ECP	Nature Reserve	E025S033BD	1	234	23			23	<i>O. (P.) zumpti</i>
AENP03	ECP	Nature Reserve	E025S033DB	2	91	5			5	<i>O. (P.) zumpti</i>
ECP01	ECP	Farm	E024S033AB	2	8	2			1	<i>O. (O.) noorsveldensis</i>
				105	5078	711	50		221	

¹ ECP = Eastern Cape Province; FSP = Free State Province; GP = Gauteng Province; LP = Limpopo Province; MP = Mpumalanga Province; NCP = Northern Cape Province; NWP = North West Province. ² QDGC = Quarter degree grid cell. ³ Three tick pools yielded ASFV genotypes la plus lb. ⁴ Two species of tick were collected on same property.

4.3 Material and methods

4.3.1 Study sites and collection of ticks

Ornithodoros ticks were collected in the same three national parks where warthog serum samples were obtained to test for antibody to ASFV [20]. The Greater Kruger National Park (GKNP) inside the controlled area was sampled as representative of an environment where circulation of ASFV in ticks and warthogs is endemic, while Addo Elephant National Park (Addo ENP) was included in the study for contrast since ASF had never been recorded in the Eastern Cape Province at the time of sampling in 2019, although there were outbreaks of the disease about 250 km east of the national park in the following year. Specific evidence of sylvatic circulation of ASFV was sought in Mokala National Park (Mokala NP) because contact with warthogs had been reported in outbreaks of the disease in domestic pigs in Northern Cape Province and adjacent western Free State Province (Figure 4.1). In addition, ticks were collected on private farms and nature reserves in seven provinces, either inside the controlled area, or in the vicinity of past outbreaks of ASF in domestic pigs outside the controlled area (Table 4.1).

The ticks were collected during 2019-2021 and each property was designated as a separate location, except for the large GKNP where multiple locations were sampled. Within locations, tick collection sites were defined as single warthog burrows or clusters of burrows sometimes overtly inter-leading but separated from other sites on the same property by distances of ≥ 1 km. Assuming a minimal ASFV infection rate of 1% the intention was to collect at least 300 ticks per location to confirm the presence or absence of virus [22], but this number could not always be attained. Coordinates of collection sites were recorded according to the quarter degree grid cell (QDGC) system [23].

Ticks of the eyeless *O. (O.) moubata* complex were collected with entomological forceps from sand scraped from the roofs, walls and floors of warthog burrows with a long-handled shovel, or from sand taken from culverts utilized by warthogs [24]. Members of the eyed *O. (O.) savignyi* complex that live in sand at animal resting sites instead of burrows, were reportedly present on four of the properties visited and attempts were made to collect them at sites indicated to be infested. Ticks were placed alive in labelled screw-top 40 ml plastic sample containers with loose filter paper liners and transported under veterinary permit in prescribed secondary and tertiary biosecurity packaging to the laboratory for examination under animal biosafety level 3 conditions. The ticks were held at laboratory temperature (22°C) for 3 weeks

prior to processing to reduce the detectability of residual virus that may have been engorged in viraemic blood meals [25].

Ticks were identified morphologically and a few specimens from each collection location were preserved in 70% ethanol as taxonomic vouchers for confirmation of identity by the Gertrud Theiler Tick Museum of the Agricultural Research Council-Onderstepoort Veterinary Research, Onderstepoort, South Africa. The remaining ticks were pooled according to sampling site, developmental instar and size into groups of 1-2 adults or 5-25 nymphs and stored at -80°C until virus and phylogenetic assays were performed.

4.3.2 Nucleic acid extraction and gene amplification

Tick pools were homogenized using 1.4 mm zirconium oxide beads in centrifuge tubes containing phosphate-buffered saline, pH7.2 (PBS) to create 10% (w/v) suspensions. Automated nucleic acid extraction was performed with IndiMag Pathogen kits (Indical Bioscience, Leipzig, Germany) using slight modifications to the manufacturer's instructions. Briefly, 200µL of tick homogenate supernatant was added to 200µL AL buffer (Qiagen), mixed well, and incubated at 70°C for 10 min. 200µL of the AL lysate was added to the IndiMag buffer for extraction. Each extraction included known ASFV positive controls.

Eluates were stored at -80°C until tested for ASFV nucleic acid using the real-time quantitative PCR (qPCR) assay of Zsak et al. [26] with modifications [27]. Briefly, 5µL DNA was amplified in 20µL reactions using 20 pmol of the published primers and 7 pmol of probe in Perfecta Fastmix II (Quanta Biosciences, Beverly, MA). Positive and no template controls (NTC) were included for each PCR run. Samples with C_q mean values ≤38 (selected as the cut-off value based on the analytical sensitivity limits of the qPCR assay) were considered positive.

To confirm the identification of ASFV detected in tick pools and classify the virus genotypes involved, nucleic acid from qPCR positive pools was amplified with primers *p72-U* and *p72-D* and the cycling conditions of Bastos et al. [10]. Appropriately-sized bands (~478 bp) of amplification product were excised from agarose electrophoresis gels, purified, and subjected to Sanger nucleotide sequencing. The sequences were viewed, 399 base fragments of DNA aligned using MEGA X software [28], and neighbour-joining phylogenetic analysis was performed with representative sequences of the 10 ASFV *p72* genotypes confirmed to occur in South Africa plus one unassigned virus (L. Heath, unpublished) using MEGA X software [28,29]

To confirm the potential significance of intra-genotypic single nucleotide polymorphisms (SNPs) observed among *p72* virus genotype I sequences, partial characterization of the central variable region (CVR) of the 9RL open reading frame of ASFV was performed on the relevant tick pool DNA extracts using the PCR primers and cycling conditions of Bastos et al. [30] followed by nucleotide sequencing of the products and deduction of amino acid sequences.

Phylogenetic characterization of ticks was based on partial sequencing of the mitochondrial 16S rRNA gene [31]. Appropriately sized bands (~313 bp) were excised from agarose electrophoresis gels, purified, and subjected to Sanger nucleotide sequencing. The sequences were viewed, 263 base fragments aligned, and neighbour-joining phylogenetic analysis performed with representative species sequences from GenBank using MEGA X software [28].

4.3.3 Isolation of virus

In attempts to isolate virus from ASFV qPCR-positive tick pools, aliquots of clarified supernatant fluid from the original 10% suspensions were inoculated at 10-fold dilutions into duplicate wells of primary pig bone marrow macrophage cultures in 96 well microplates, and examined for hemadsorption (HAD) and cytopathic effect (CPE) at 48 and 72 h incubation [32]. Negative samples were scheduled to be sub-cultured twice, and isolation of virus in HAD- or CPE-positive samples confirmed by performing qPCR [27] on culture extracts.

4.3.4 Identification of tick blood meal donor species

In attempts to identify blood meal donors, partial cytochrome b sequences of mammalian mitochondrial DNA were determined from selected suspensions of pools of engorged ticks [33] and compared by BLASTx search with sequences in GenBank (NCBI).

4.3.5 ASFV antibody tests on alternative vertebrate hosts of *Ornithodoros* ticks

Dried blood samples were collected on Nobuto cellulose strips (NCS) (Advantec, Tokyo, Japan) from 5 leopard tortoises (*Stigmochelys pardalis*) killed in road accidents in the Kimberley area of Northern Cape Province and in the southern GKNP in 2020-2021. Serum samples from 97 spotted hyaenas (*Crocuta crocuta*) obtained from the GKNP came from animals translocated internally or culled for managerial purposes in the south of the national park from 1997 to 2018 and had been stored at -80°C. Tests for antibody to ASFV were performed on the blood and serum and samples with INgezim PPA Compac R. 11.PPA.K3

blocking enzyme-linked immunosorbent assay (ELISA) kits (Eurofins Technologies Ingenasa, Madrid, Spain) as described previously [20].

4.4 Results

4.4.1 Collection and identification of *Ornithodoros* ticks

A total of 5,078 ticks was collected at 82 sites in 45 locations in 7/9 provinces of South Africa during 2019-2021. An additional 23 sites in 14 locations failed to yield ticks, including a nature reserve in Mpumalanga Province bordering Gauteng Province, plus 7 small contiguous farms in north-eastern GP where acaricides were used liberally, and 6 farms in western Gauteng Province where warthogs were rarely seen, and the burrows appeared to be occupied by porcupines (Table 4.1). Ticks were identified morphologically, and a subset of 436 ticks was preserved as taxonomic vouchers. The remaining 4,642 ticks were assayed in 711 pools for virus content.

Partial mitochondrial 16S rRNA gene sequences were determined for 221 tick pools, including pools that tested positive for ASFV nucleic acid plus at least one pool per collection location and all pools from some locations, except for location LP02 where inadequate DNA remained available (GenBank accession numbers MZ411417-MZ411419, OK136965-OK137179, OK323967, OK323968 and OL870945) (Table 4.1).

All 221 partial mitochondrial 16S rRNA tick gene sequences were aligned and subjected to neighbour-joining phylogenetic analysis [28] with 74 corresponding gene sequences obtained from GenBank for Afrotropical *Ornithodoros* ticks, mainly from South Africa but including *O. (O.) porcinus* Walton, 1962 from Tanzania and *O. (O.) savignyi* (Audouin, 1826) from Sudan, plus the ixodid tick *Amblyomma hebraeum* as an outlier. The 221 data sets from the present study resolved into 11 unique sequences while the 74 data sets from GenBank represented 19 unique *Ornithodoros* sequences, corresponding to a collated total of 23 unique sequences clustering as 10 currently recognized species of *Ornithodoros* ticks [21] (data not shown). For sake of clarity the analysis was repeated using only representative unique sequences from the present study and from the GenBank data, resulting in the generation of a dendrogram showing the same topology and phylogenetic relationships as the full range of data sets (Figure 4.2).

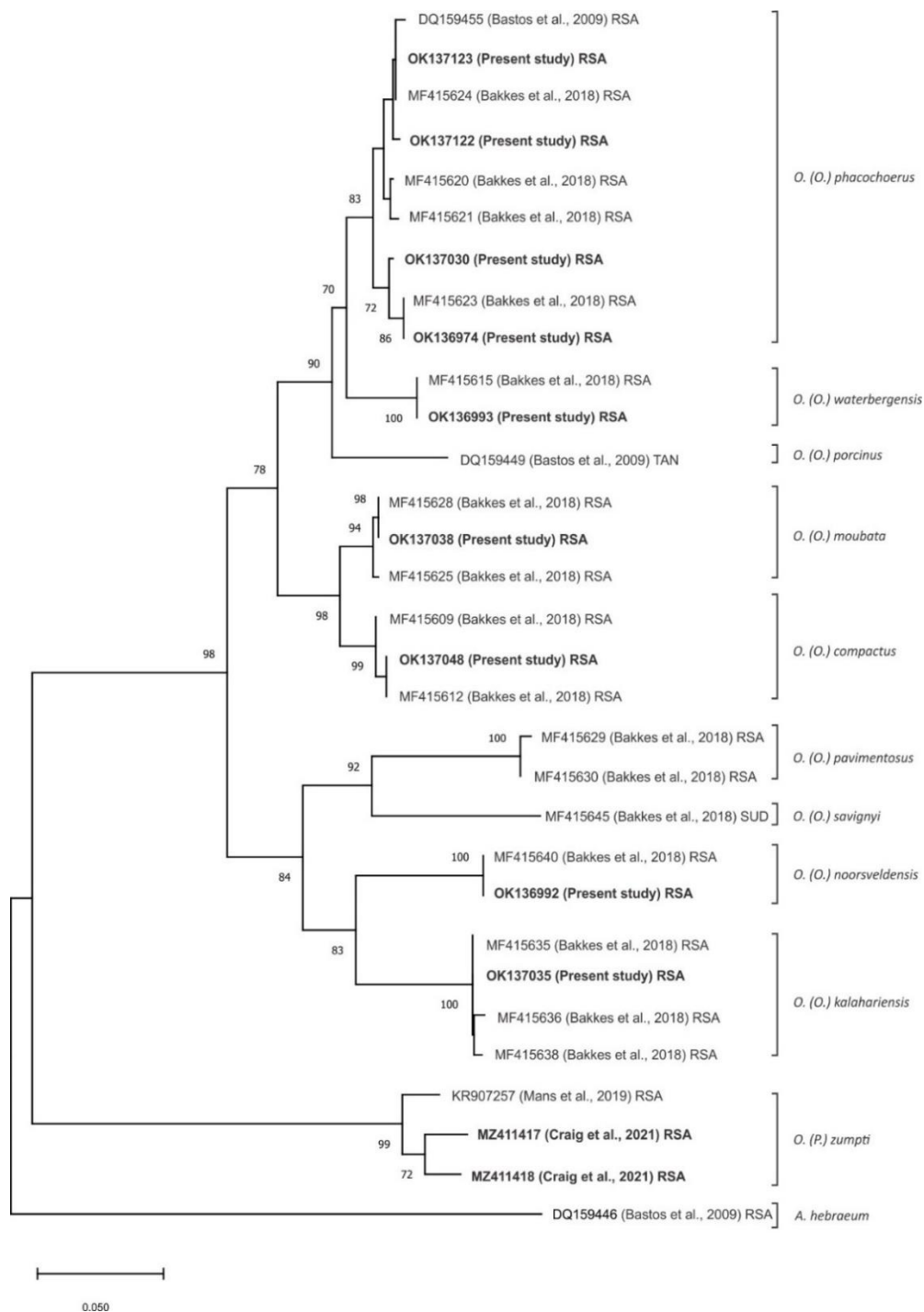


Figure 4.2: Neighbour-joining tree based on partial mitochondrial 16S rRNA gene sequence (263 nt) depicting phylogenetic relationships between 11 unique sequences generated from African *Ornithodoros* (*Ornithodoros*) ticks in the present study (GenBank accession numbers in bold) and 19 representative unique *Ornithodoros* species sequences from Genbank, mainly from South Africa (RSA) but including *O. (O.) porcinus* Walton, 1962 from Tanzania (TAN) and *O. (O.) savignyi* (Audouin, 1826) from Sudan (SUD), plus the ixodid tick *Amblyomma hebraeum* as an outlier. Percentage bootstrap support values were derived following 10,000 replications. Evolutionary analyses were conducted in MEGA X.

The ticks collected in the present study were found to include all four species of the *O. (O.) moubata* complex known to occur in South Africa: *O. (O.) moubata* (Murray) (1877) sensu Walton, 1962, *O. (O.) waterbergensis* Bakkes et al., 2018, *O. (O.) phacochoerus* Bakkes et al., 2018 and *O. (O.) compactus* Walton, 1962, each within a distinct distribution range (Table 4.1). Furthermore, two of the three species of the *O. (O.) savignyi* complex ticks known to be present in the country, *O. (O.) kalahariensis* Bakkes et al., 2018 and *O. (O.) noorsveldensis* Bakkes et al., 2018, were found at single locations in Northern Cape Province and Eastern Cape Province. In addition, the only member of the subgenus *Pavlovskyella* yet recorded in South Africa, *O. (P.) zumpti* Heisch and Guggisberg, 1953, was collected from warthog burrows and a yellow mongoose (*Cynictis penicillata*) burrow in Addo ENP in Eastern Cape Province (Table 4.1).

4.4.2 Detection and *p72* phylogeny of ASFV nucleic acid in ticks

Of the 711 pools of ticks screened by qPCR for ASFV nucleic acid, 50 pools tested positive with a Cq mean value of 26.00 (range 20.23-36.11), including members of all four of the *O. (O.) moubata* complex sampled. A further 10 pools of ticks produced doubtful reactions in the qPCR and failed to react in the *p72* PCR or could not be sequenced, and the findings were discarded since the presence of virus was already confirmed in other tick pools at the same collection sites. As expected, the few *O. (O.) kalahariensis* and *O. (O.) noorsveldensis* ticks of the *O. (O.) savignyi* complex that do not live in burrows failed to yield ASFV nucleic acid, as did the *O. (P.) zumpti* ticks from Eastern Cape Province where the presence of the virus had never been recorded at the time that the ticks were collected (Table 4.1). The locations where ASFV nucleic acid was detected in ticks collected during the present study are shown in Figure 4.3 in relation to locations where ASFV was not found in ticks.

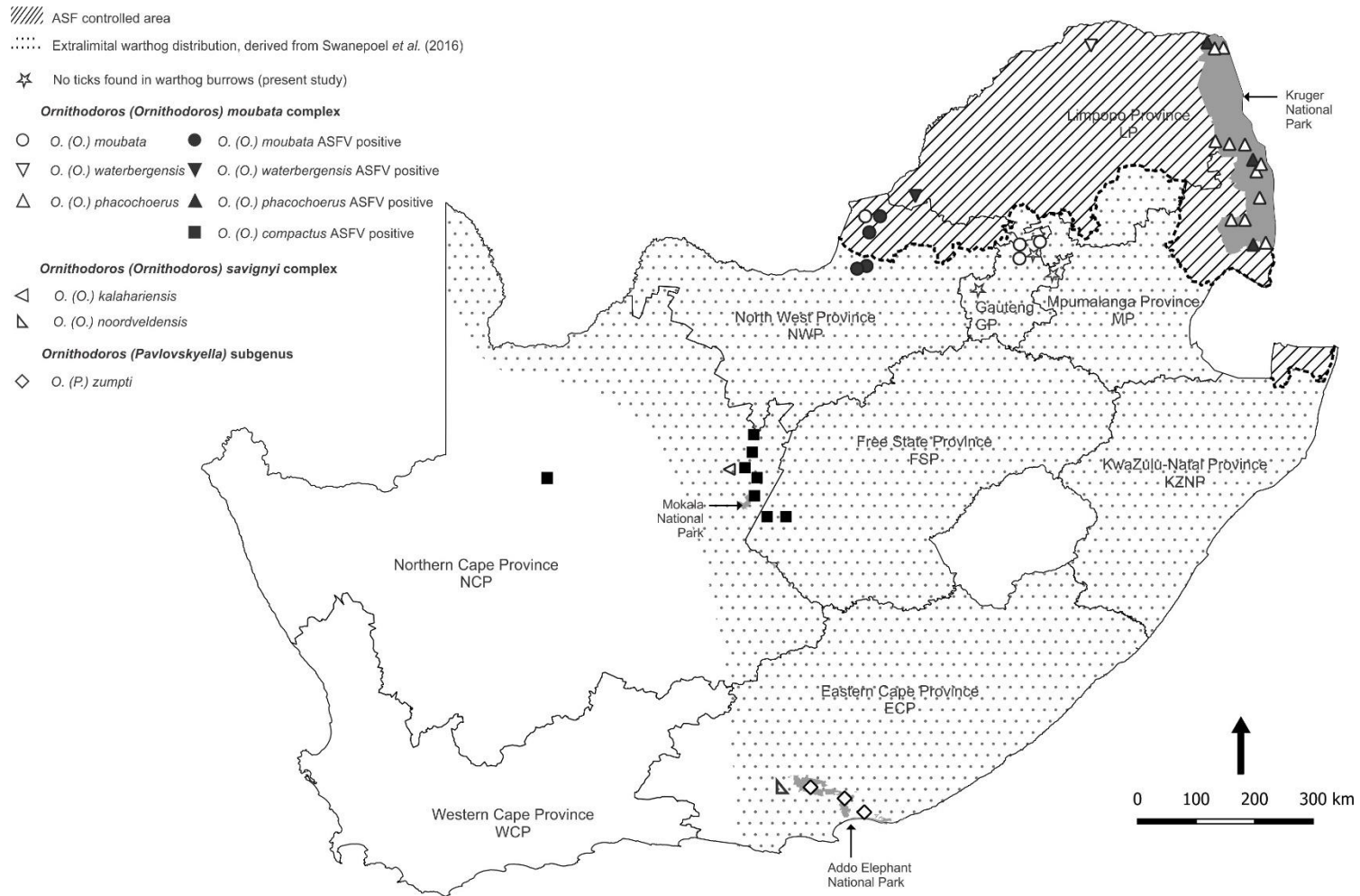


Figure 4.3: Spatial distribution of locations where different *Ornithodoros* tick species were collected during the present study. Locations where African swine fever virus nucleic acid was detected in ticks (closed symbols) are shown in relation to locations where viral nucleic acid was not found in ticks (open symbols).

All 50 of the qPCR-positive tick pools also tested positive in the *p72* PCR and phylogenetic analysis revealed that the ASFV nucleic acid detected in the ticks represented five *p72* genotypes of the virus (Figure 4.4; Table 4.1), with SNPs that distinguished 4 variants of genotype I supported by marked size differences in the CVR compatible with subtypes recorded in recent outbreaks of the disease in domestic pigs outside the controlled area [13]; see *Discussion* below. Only 20 sequences from the present study were included in the analysis, comprising one unique partial *p72* sequence per tick species per collection site plus one sequence (GenBank accession number OL415194) from a warthog [20], along with 29 representative sequences of the genotypes known to occur in South Africa including isolate Spencer that has not been assigned to a *p72* genotype; see *Discussion* below. The inclusion of a type II isolate (RSA 08/2019, Figure 4.4) (L. Heath, unpublished) in the analysis incidentally raises the number of known genotypes confirmed to be present in South Africa to 10, but an update on ASFV isolations from recent outbreaks of the disease will be presented separately. In the present study, ticks of the *O. (O.) waterbergensis* and *O. (O.) phacochoerus* species yielded ASFV genotypes XX, XXI, XXII at 4 locations and *O. (O.) moubata* yielded 2 subtypes of ASFV genotype I at two locations inside the controlled area. Outside the controlled area, *O. (O.) moubata* and *O. (O.) compactus* ticks yielded 4 subtypes of ASFV genotype I variously at 7 locations with 3 pools of *O. (O.) compactus* ticks yielding two subtypes, Ia and Ib, of genotype I at location NCP01. In addition, genotype III ASFV was identified in *O. (O.) compactus* ticks at a single location outside the controlled area in western Free State Province (GenBank accession numbers OK148637-OK148688 and OM135580) (Table 4.1). Two separate GenBank submissions were made for each of three tick pools that yielded subtypes Ia and Ib of genotype I ASFV (Table 4.1). The detection of genotype III virus outside the controlled area was confirmed by re-extraction of an aliquot of the original tick suspension and repeating the partial characterization of the *p72* gene.

The distribution patterns of the ASFV genotypes identified in ticks during the present study are summarized on a regional basis in Figure 4.5 in relation to virus genotypes identified in outbreaks of ASF in domestic pigs during 2016-2021 [6-9,12,12,34]. The extant information on the distribution ranges of the currently recognized species of *Ornithodoros* ticks in South Africa is collated in Figure 4.6 by plotting the locations where ticks were collected during the present study in relation to locations where ticks with cognate partial mitochondrial 16S RNA gene sequences or morphological identity were previously reported [21,31,35-38].

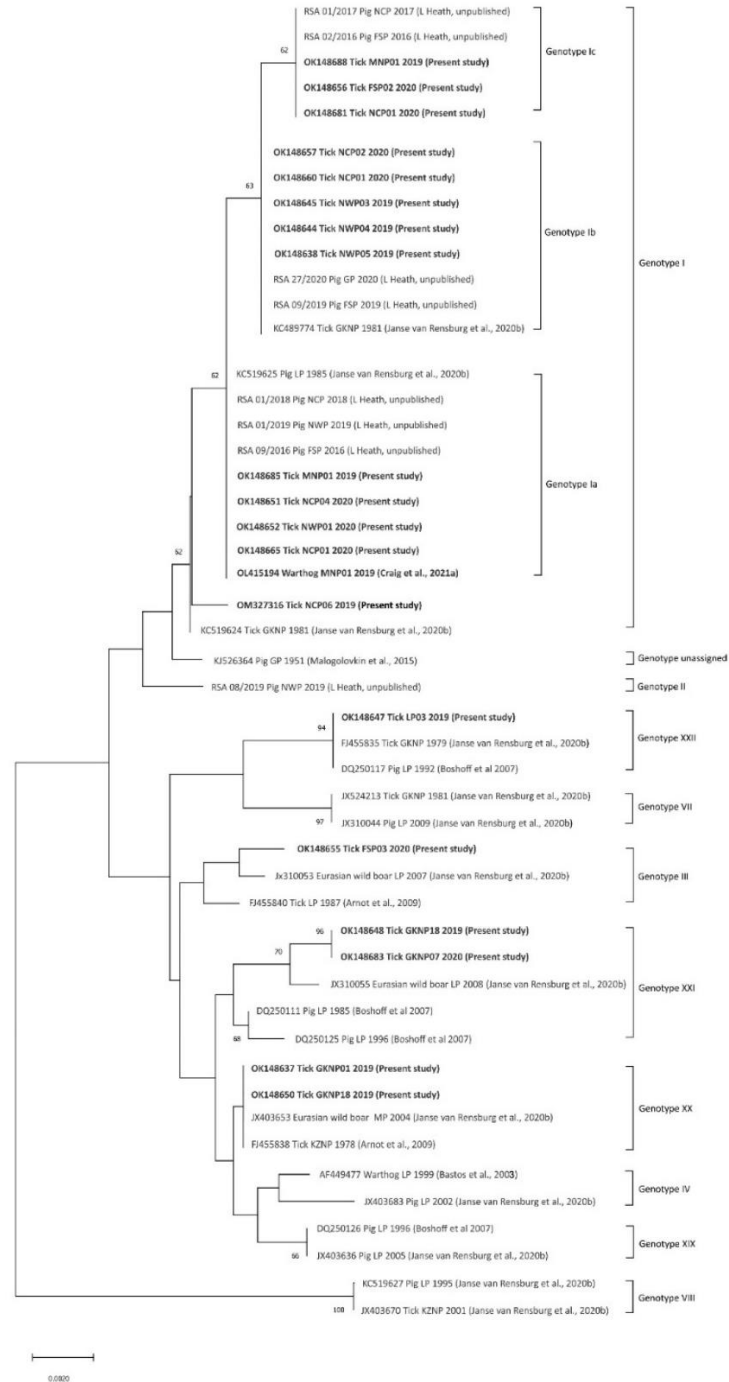


Figure 4.4: Neighbour-joining tree based on partial C-terminal *p72* gene sequence (399 nt) of African swine fever virus depicting phylogenetic relationships between 20 representative sequences detected in African *Ornithodoros* (*Ornithodoros*) ticks in the present study (GenBank accession numbers in bold) and 29 representative sequences of the genotypes known to occur in South Africa. Only one unique sequence detected per tick species per collection site in the present study is included in the analysis and these comprise 5 genotypes of virus. Percentage bootstrap support values were derived following 10,000 replications. Evolutionary analyses were conducted in MEGA X.

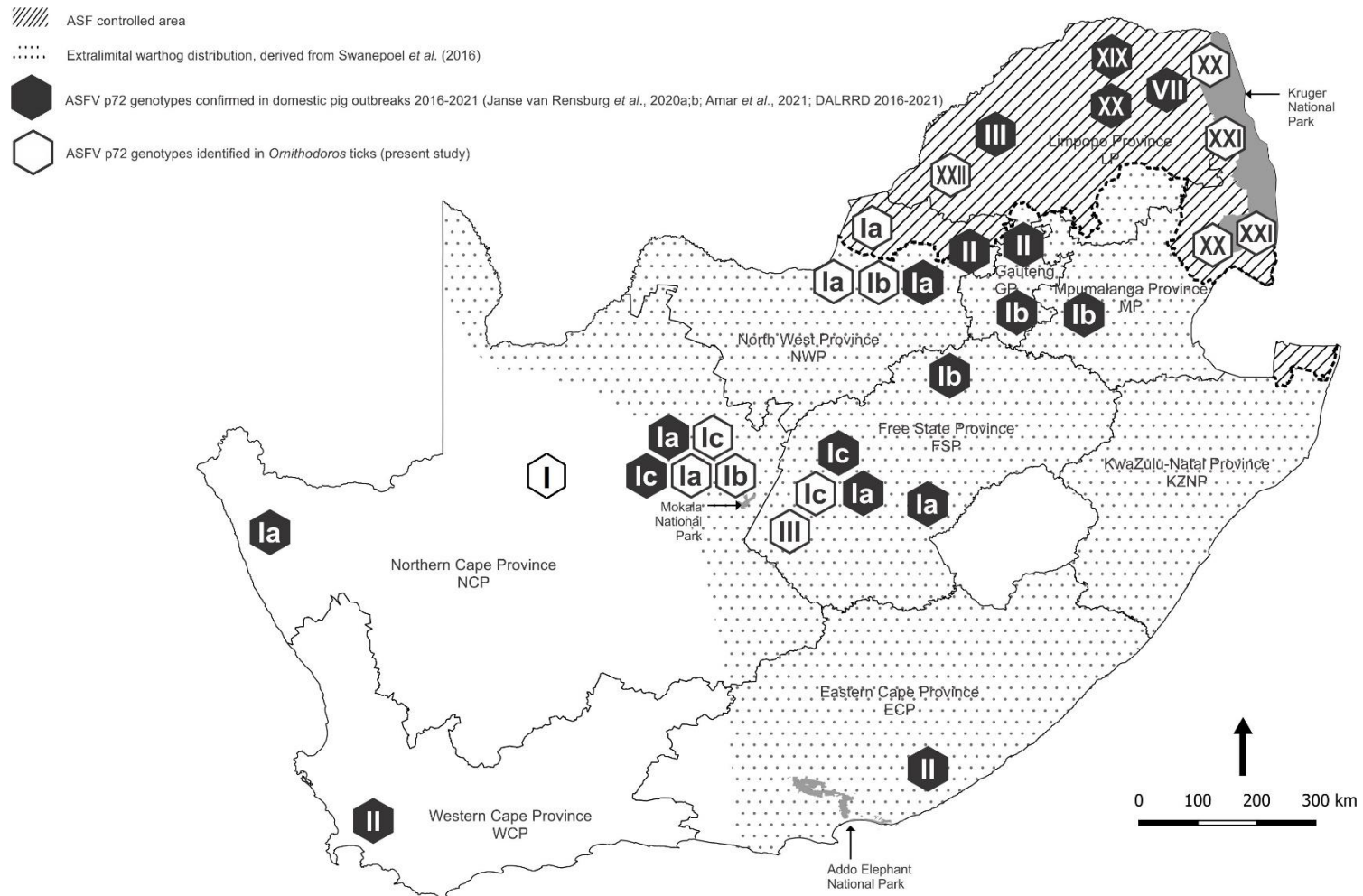


Figure 4.5: Spatial distribution of African swine fever virus genotypes identified in ticks during the present study (open symbols), summarized on a regional basis, in relation to distribution of virus genotypes identified in outbreaks of African swine fever in domestic pigs (closed symbols) during 2016-2021.

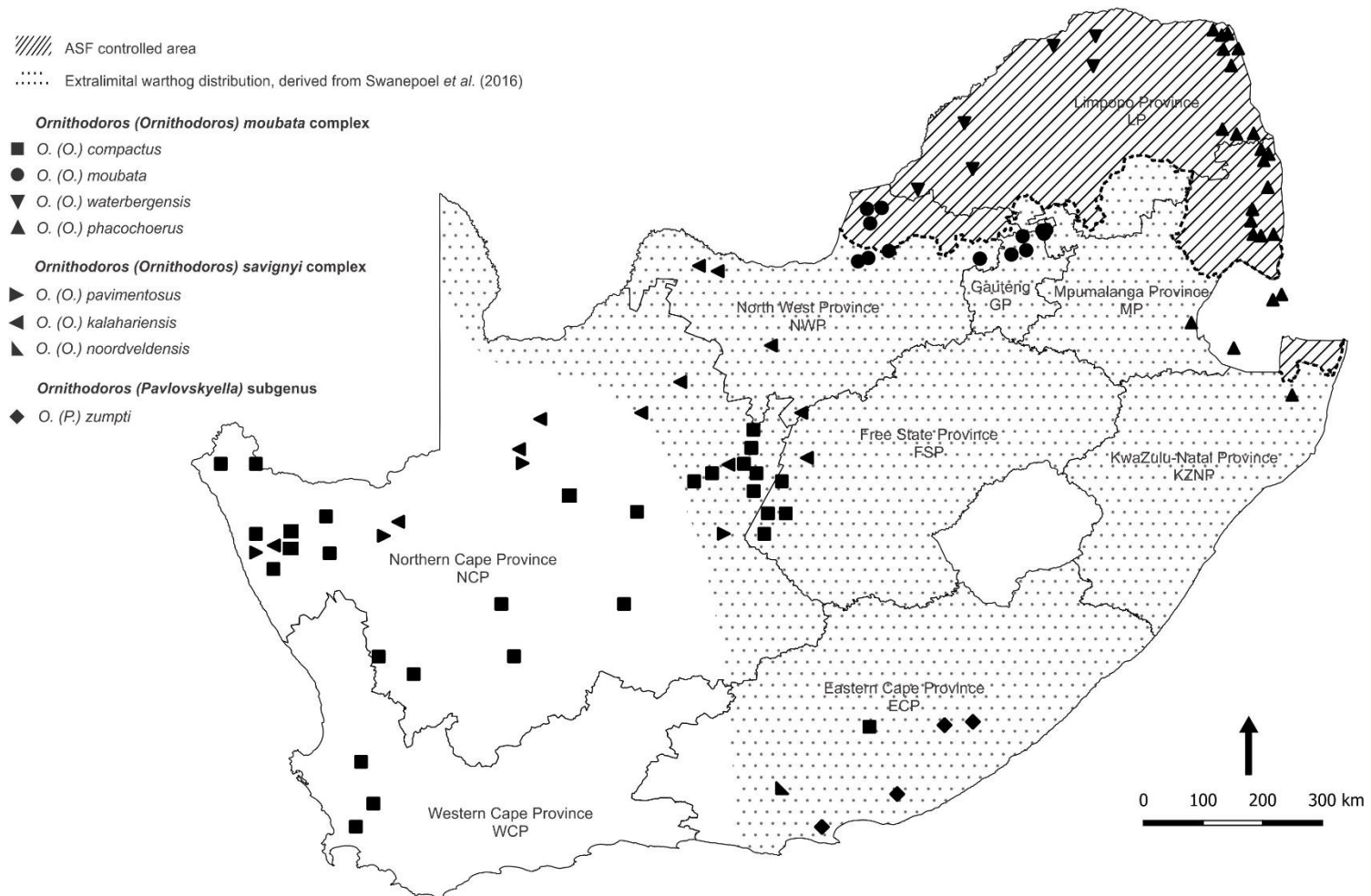


Figure 4.6: Updated distribution ranges of the currently recognized species of *Ornithodoros* ticks in South Africa based on locations where ticks were collected during the present study together with locations where ticks with cognate partial mitochondrial 16S RNA gene sequences or morphological identity were previously reported by sources cited in the text.

4.4.3 Isolation of virus

In an initial attempt to isolate virus from ASFV qPCR-positive tick pools, 6/18 samples produced positive HAD results on first pass in cell cultures, but further investigations had to be deferred since the preparation of macrophage cultures was suspended during the COVID-19 pandemic. From qPCR performed on laboratory stock ASFV that is routinely titrated as a control with each batch of samples cultured for virus isolation, it was extrapolated that tick suspensions that had qPCR Cq values of ≤ 22 probably had infective titres $\geq 10^{6.0}$ HAD₅₀/ml. This applied to at least one tick pool from each of the *O. (O.) phacochoerus*, *moubata* and *compactus* samples tested, while the single *O. (O.) waterbergensis* qPCR-positive tick suspension had an extrapolated titre of $\geq 10^{4.0}$ HAD₅₀/ml.

4.4.4 Molecular identification of blood meal hosts

Determination of partial cytochrome b sequences of mammalian mitochondrial DNA [33] was attempted on suspensions of 64 pools of partially engorged ticks, including pools that yielded ASFV nucleic acid, but only one pool of *O. (O.) phacochoerus* ticks from collection location GKNP01 and one pool of *O. (P.) zumpti* ticks from location AENP02 produced sequences, and a BLASTx search of GenBank (NCBI) confirmed that warthogs served as blood meal donors for ticks in both pools.

4.4.5 ASFV antibody tests on hyaena and tortoise sera

Dried blood samples from 2 leopard tortoises (*Stigmochelys pardalis*) collected in southern GKNP and 3 collected in the Kimberley area of Northern Cape Province plus 97 serum samples from spotted hyaenas in the GKNP all tested negative for antibody to ASFV.

4.5 Discussion

The repeated detection of viral nucleic acid in *Ornithodoros* ticks and the high prevalence of antibody observed in the sera of extralimital warthogs [20] are suggestive of extensive sylvatic circulation of ASFV beyond the controlled area in South Africa that warrants further investigation. However, the full implications of the present findings are best assessed in relation to the extant information on sylvatic ASFV.

There are over 100 known species of *Ornithodoros* ticks worldwide; the exact number varies with periodic discoveries and taxonomic revisions [21,38-41]. The resistance to desiccation conferred by the wax and cement layers of their cuticles facilitates their occurrence in the warmer and more arid regions of all continents [42-44]. The ticks are nidicolous, living in close

association with their hosts. Distinction is made between endophilous nidicoles such as *O. moubata* sensu lato that occur within host habitations including animal burrows, and harbourage nidicoles such as *O. savignyi* s.l. that live in the environs of their hosts, for example in sand at animal resting sites, although differences in behaviour are not always clear-cut [43,45].

Several African and exotic *Ornithodoros* species have been found capable of sustaining replication and transmission of ASFV experimentally, but vector competence appears to be regulated by multiple tick species- and virus strain-specific factors [46-73]. Hence, it is important that both ticks and virus should be identified accurately in assessing and recording vector potential.

Endophilous Afrotropical *Ornithodoros* species adapt readily to living in the cracks and crevices of rustic human dwellings and livestock shelters, and this influenced the manner in which the taxonomy of the ticks evolved (Table 4.2). The type species of the genus *Ornithodoros*, *O. savignyi* (Audouin, 1827), has eyes and was originally described as *Argas savignyi* Audouin, 1827 from specimens collected from sand in Egypt (Table 4.2) [74]. The eyeless *O. moubata* (Murray) (1877), sensu Walton, 1962, was described as *Argas moubata* Murray, 1877 from a specimen collected in Angola where the presence of the ticks in human dwellings had been noted many years earlier [75]. Both species were subsequently transferred to the genus *Ornithodoros* that was erected by Koch [76] and is sometimes rendered incorrectly as *Ornithodorus* [77]. There was also description of an eyeless *O. savignyi* var. *caeca* Neumann (1901), occasionally reported as *caecus* or *caecum*, but this was soon synonymized with *O. moubata* [78] and is omitted from Table 4.2. An additional eyed species, *O. (O.) pavimentosus* Neumann, 1901, was described from a single specimen from Namibia but later synonymized with *O. savignyi* [79], and subsequently resurrected with re-description based on neotype specimens from Northern Cape Province, South Africa [21].

By the late nineteenth and early twentieth century *Ornithodoros* ticks were widely known to inhabitants of Africa as infesting dwellings and inflicting painful bites while people slept at night, sometimes transmitting a potentially fatal illness that proved to be tick-borne relapsing fever (TBRF) caused by borrelias [75,80-84]. Investigators of TBRF routinely distinguished between *O. savignyi* s.l. and *O. moubata* s.l. based on the presence or absence of eyes.

Table 4.2: Abridged evolution of the taxonomy of the Afrotropical members of the subgenus *Ornithodoros* (*Ornithodoros*) (*Acari: Ixodida: Argasidae: Ornithodorinae*).

Original description	Walton, 1962	Van der Merwe, 1968	Walton, 1979	Bakkes et al., 2018 (Type locality)
Eyes absent: <i>Ornithodoros moubata</i> group/complex				
<i>Argas moubata</i> Murray, 1877	<i>O. moubata</i>	<i>O. moubata moubata</i>	<i>O. moubata</i>	<i>O. moubata</i> (South Africa)
	<i>O. porcinus porcinus</i>	<i>O. moubata porcinus</i>	<i>O. porcinus porcinus</i>	<i>O. porcinus</i> (Tanzania)
				<i>O. phacochoerus</i> (South Africa)
				<i>O. waterbergensis</i> (South Africa)
	<i>O. porcinus. domesticus</i>		<i>O. porcinus domesticus</i>	
			<i>O. porcinus avivora</i>	
	<i>O. apertus</i>	<i>O. moubata apertus</i>	<i>O. apertus</i>	<i>O. apertus</i> (Kenya)
	<i>O. compactus</i>	<i>O. compactus</i>	<i>O. compactus</i>	<i>O. compactus</i> (South Africa)
Eyes present: <i>Ornithodoros savignyi</i> group/complex				
<i>Argas savignyi</i> Audouin, 1827*	<i>O. savignyi</i>	<i>O. savignyi</i>	<i>O. savignyi</i>	<i>O. savignyi</i> (Egypt)
<i>O. pavimentosus</i> , Neumann, 1901†				<i>O. pavimentosus</i> (South Africa)
				<i>O. kalahariensis</i> (South Africa)
				<i>O. noorsveldensis</i> (South Africa)

*Subsequently transferred to genus *Ornithodoros*, Koch 1844

†Synonymized with *O. savignyi*, Theiler and Hoogstraal, 1955; resurrected, Bakkes et al., 2018.

Although it was known that livestock sharing human dwellings at night were also bitten, *O. moubata* s.l. was considered to be primarily a parasite of humans, prevalent from Eritrea in the north to as far south as Graaff-Reinet in South Africa, and including present day Ethiopia, South Sudan, Somalia, Uganda, Kenya, Tanzania, Malawi, Mozambique, Zambia, Zimbabwe, Namibia, Botswana, Angola and Madagascar, with incursion westwards into the Democratic Republic of the Congo (DRC), Congo Republic and Cameroon along trade routes [75,85-90].

Transmission of ASFV by *O. (P.) erraticus* ticks was discovered in 1963 in Spain following accidental introduction of the virus into Europe [91] and prior to this event veterinary interest in *Ornithodoros* ticks was relatively limited in Africa. Nevertheless, on various occasions from 1907 to 1943 the presence of *O. moubata* s.l. ticks was recorded in pigsties in Angola, DRC, Zimbabwe, South Africa and Malawi, warthog burrows in Zambia and DRC, on a warthog shot in Uganda, in association with poultry and other domestic animals in South Africa and, notably, was collected as *Argas moubata* from 44 tortoises in the environs of Niekerkshoop in Northern Cape Province, South Africa [92-101]. The investigators of TBRF were prompted to explore the possibility that *O. moubata* s.l. had sylvatic origins, possibly in association with large burrowing animals such as warthogs and porcupines [90,102,103]. Evidence emerged that *O. moubata* s.l. had also been found on elephant, lion, hyaena, ground pangolin (*Smutsia temminckii*), antbear (*Orycteropus afer*) and domestic cattle in addition to warthogs and porcupines in Angola and Mozambique [104-106]. Walton [90,103,105-108] collected *O. moubata* s.l. ticks from different locations and domestic or wild habitats and compared the duration of their life cycles and longevity under laboratory conditions to identify a range of 'biological forms' that implied the taxon was not monotypic. Limited observations on the viability and teratology of hybrids tended to support the distinctness of the biological forms.

Walton [105,109] noted that the designation *O. moubata* (Murray) 1877 was a nomen dubium since the description was inadequate and the type specimen was lost, and he replaced it with four species and a subspecies derived from the biological forms that he had identified (Table 2). He used ticks of the 'Groot Marico strain' derived from human dwellings on a property (location NWP02, Table 4.1) north of a village of that name in South Africa as neotypes for re-description of *O. moubata* since they were found to be 'identical biologically' to ticks from Angola and Namibia. The re-described *O. moubata* was regarded as being associated particularly with human dwellings in southern Africa but was stated to range in distribution north-eastwards to Tanzania, while the possibility and extent of its occurrence in wild habitats was considered speculative [106]. A new species, *O. porcinus*, was erected for ticks associated with warthog burrows in East Africa, but its distribution was postulated to extend to southern Africa, while a new subspecies, *O. porcinus domesticus*, was described as highly

prevalent in human dwellings in East Africa [105,106]. The two remaining new species of Walton were considered to be limited to wild habitats; *O. apertus* associated with porcupine (probably *Hystrix cristata*) burrows in Kenya and *O. compactus* found on tortoises in the south-western region of South Africa, while *O. savignyi* was retained as valid [105,106]. The utility of Walton's qualitative morphological descriptions and the validity of his biological observations were questioned by van der Merwe [110] who proceeded to merge the subspecies *O. p. domesticus* with *O. p. porcinus*, and re-assigned the taxa *moubata*, *porcinus* and *apertus* as subspecies of *O. moubata*, but retained *O. compactus* as a distinct species. However, Walton [111] re-affirmed his classification of the *O. moubata* complex and added a further subspecies, *O. p. avivora*, that parasitized domestic chickens along the coast of East Africa (Table 4.2).

Public health measures and the use of increasingly effective antibiotics and acaricides ultimately led to control of TBRF although it remains a threat [75,105,106,112-114], while interest in ASFV intensified. Black and Piesman [115] applied phylogenetic analysis based on partial sequencing of the mitochondrial 16S rRNA gene to ixodid and argasid ticks, and the incorporation of this approach into ASFV investigations revealed the existence of three geographically discrete lineages of *O. porcinus* s.l. but provided no support for recognition of *O. p. porcinus* and *O. p. domesticus* subspecies [31,36,37]. Bakkes et al. [21] duly undertook taxonomic revision of the Afrotropical *Ornithodoros* (*Ornithodoros*) subgenus and since the ticks are lacking in highly variable physical features useful for delimitation of species, they resorted to morphometric analysis of the profile of dorsal protuberances on the tarsal segment of the first leg to distinguish species that were corroborated by the lineages they generated in phylogenetic analysis. The *O. moubata* species of Walton [105] was retained as a parasite of warthogs, other burrowing animals, livestock and humans in north-western South Africa and adjacent countries, but historic records from East Africa were regarded as unconfirmed. Similarly, *O. porcinus* was retained as parasitizing warthogs, other wildlife, humans and livestock in East Africa. A new species, *O. phacochoerus*, was described as being associated with warthogs, livestock and humans in eastern South Africa and contiguous countries, while *O. waterbergensis* was erected as a new species with a similar host range in north-western South Africa. The *O. apertus* species of Walton was retained as being associated with porcupines in East Africa, and *O. compactus* as occurring on tortoises in south-western South Africa. Bakkes et al. [21] postulated that *O. savignyi* sensu stricto is restricted to northern Africa and the Near East, and they replaced it in the south-west of the African continent with a new species *O. kalahariensis* described as partially sympatric with *O. pavementosus*, a species that they resurrected from synonymy. Finally, they erected *O. noorsveldensis* as a new eyed species known from a single locality in Eastern Cape Province, South Africa

(location ECP01, Table 4.2). Since members of the *O. savignyi* complex are not associated with burrows it has been surmised that they are not involved in sylvatic circulation of ASFV, although *O. savignyi* s.s. was shown to be capable of transmitting the virus experimentally [24,50,116].

The identities of ticks collected during the present study are consistent with the classification of Bakkes et al. [21] and extend the known distribution ranges of their eyeless species in congruent manner, but we did not determine geographic limits of occurrence or areas of sympatry (Table 4.1; Figures 4.2; 4.6). Although morphometric analysis can be impractical for use in large scale surveys, ASFV vector studies usually involve molecular assay of individual ticks for virus content that allows for convenient incorporation of tick phylogenetics [31]. Hence, the taxonomic classification of Bakkes et al. [21] should find general application, particularly since they anticipated that further studies could reveal additional novel species of ticks.

The distribution ranges of *Ornithodoros* ticks in South Africa as plotted in Figure 4.6 incorporate sites generated in the present study along with those given by Bakkes et al. [21] including records in their supplementary information, as well as records given by the same team in Mans et al. [38], and sites deduced by relating sequence data from past publications [31,36,37] to the current taxonomy. The sea bird parasite *Ornithodoros (Alectorobius) capensis* Neumann, 1901 was excluded as irrelevant to the present study although it would probably feed readily on any host species that impinged on its specialized habitats [43,45].

It is notable that *O. compactus* was described as a novel species by Walton [105] based on ‘*Argas moubata*’ specimens collected in 1932 from 44 tortoises of two species in Niekerkshoop, South Africa, as reported by Bedford [98], plus additional specimens from tortoises in Northern Cape Province, South Africa, as well as ticks collected from a tortoise in Hamburg Zoological Gardens and from the Reptile House in Regent’s Park Zoological Gardens, London. The same tick species was found in ‘tortoise burrows’ at two sites in Northern Cape Province, South Africa, (supplementary information) [21,38]. Moreover, 507 *O. compactus* ticks, including some adults, were reportedly found on 55 tortoises of seven species and subspecies, mainly in Western Cape and Northern Cape Provinces, South Africa, but also in adjacent Eastern Cape and Free State Provinces [35]. The ticks apparently lodge in the axial and perineal skin folds of tortoises [105]. Since all distribution records of *O. compactus* prior to the present study relate at least indirectly to tortoises, we have accepted the distribution records of Horak et al. [35] and incorporated them in Figure 4.6.

We not only found *O. compactus* to occur commonly in warthog burrows in Northern Cape Province and western Free State Province, South Africa, but detected a high prevalence of ASFV in these ticks, including virus nucleic acid in 19/51 pools tested from location NCP01 (Table 4.1). We were prompted to investigate whether tortoises might be involved in circulation of ASFV, but managed to obtain only 5 dried blood samples from road kills that carried no *Ornithodoros* ticks and tested negative for ASFV nucleic acid and antibody. There are approximately 50 species of terrestrial tortoises in existence worldwide, with Africa being particularly rich in diversity. Some 14 species and subspecies of tortoises occur in the south-western region of the African continent with 12 of them being endemic, including threatened and endangered species subject to illegal international trade [117-119]. Even if tortoises prove to be refractory to ASFV infection, and despite their slow mobility, they could carry infected ticks long distances by air and other means of transport, which constitutes good reason to strengthen control of the illegal trade in endangered animals like tortoises and pangolins. Furthermore, the observations on *O. compactus* reinforce the perception that *Ornithodoros* ticks are facultative with regard to blood meal donors and habitat, so that naming of tick species for putative hosts could be misleading.

The hyaena sera were tested in parallel with the tortoise blood samples to check whether non-suids that are potentially exposed to ASFV infection, as in *Ornithodoros* tick infested burrows or culverts, produce detectable antibodies irrespective of their ability to sustain replication of the virus. Although previous investigators also failed to detect antibody to ASFV in non-suids, the numbers of samples that have been examined are relatively limited and the tests used in early studies were less sensitive than the blocking ELISA [120]. The poor results obtained in attempts to identify donors of blood meals probably relate to the fact that the test used is better suited to dipteran vectors, and improved techniques are available for use on ticks, while testing for antibody to *Ornithodoros* salivary antigens would have constituted a useful screening method to identify potential hosts of the ticks [121,122].

Although the high prevalence of antibody in warthogs [20] and presence of virus in ticks outside the controlled area in South Africa are consistent with sylvatic circulation of ASFV [3,24,116,123], definitive vector competence studies remain desirable, particularly for *O. (P.) zumpti* ticks, but lay beyond the scope of the present project. A short communication on finding *O. (P.) zumpti* ticks in warthog burrows was published separately to stimulate field investigations in the vicinity of ongoing outbreaks of ASFV infection in Eastern Cape Province [124]. Other ticks of the subgenus *Pavlovskyella* known to be present in Africa comprise members of the *O. (P.) erraticus* complex, including *O. (P.) maroccanus* and *sonrai* that have distribution ranges extending from the Iberian Peninsula to the north African littoral and West

Africa. The occurrence of ASFV nucleic acid was demonstrated in *O. (P.) sonrai* ticks collected in proximity to pigsties in Senegal, but the epidemiological significance of the finding was considered doubtful [125].

Viruses from the present study will be fully characterized as circumstances permit. Meanwhile, it is notable that SNPs observed among partial *p72* sequences of genotype I isolates in South Africa were stable within recent series of outbreaks of disease in pigs connected by spread of infection [13], and were supported by differences in product sizes of the CVR of the 9RL open reading frame of ASFV (L. Heath, unpublished). It is clear in retrospect that variants of genotype I had been isolated decades earlier from outbreaks of disease in pigs and from ticks, putatively *O. (O.) phacochoerus*, collected in GKNP in 1981 (Figure 4.4) [13,34]. Preliminary designation of some of the variants as subtypes Ia, b and c [13] should not be confused with the same designations applied to ASFV genotypes defined by *p54* gene sequences [126], and the ambiguity will be addressed in a fuller description of the phenomenon. The present recovery of multiple subtypes of genotype I ASFV from ticks collected at single locations (Table 4.1), even from the same burrows, appears to be a novel observation.

Virus (GenBank accession number OM135580) identified in *O. compactus* ticks at location NCP06 (Table 4.1; Figures 4.3; 4.5), which lies beyond the western limit of the known distribution of warthogs as plotted in 2016 [18], may represent a further variant of genotype I, closest to a 1981 tick isolate from GKNP (Figure 4.4). Apparently, the presence of warthogs on property NCP06 relates to translocations made to a neighbouring property during the 1990s. Many unrecorded transfers of warthogs were made to private properties following the original translocations to nature reserves in the south of the country conducted by conservation officials during the 1960s and 1970s [17,127]. Interestingly, isolate Spencer that does not correspond to any existing *p72* genotype and is represented in Figure 4.4 by GenBank accession number KJ526364 [128,129], was originally obtained in 1951 from an outbreak of disease in southern Gauteng Province following the introduction of infection from Namibia [130,131]. Hence, its close relationship to recent Namibian isolates is understandable [132].

The detection of genotype III ASFV in ticks at a single location outside the controlled area (FSP03, Table 4.1) is difficult to explain. This is a small property in western Free State Province where few ticks were found in a single cluster of warthog burrows and there was no history of the disease in pigs kept in a fully enclosed building. Genotype III ASFV had previously been found in outbreaks of disease in pigs and in ticks at several locations only within the controlled area between 1993 and 2017 [34]. In contrast, the range of viruses

detected in ticks collected within the controlled area in the present study (Table 4.1) is typical of the variety of genotypes of ASFV known to circulate in areas where sylvatic circulation of ASFV is endemic [14].

Subtype 1c of ASFV was identified in *O. (O.) compactus* ticks collected from burrows on property FSP02 (Figures 4.1; 4.3; Table 4.1) outside the controlled area in western Free State Province where the same subtype had been identified in 2016 in an outbreak of disease in free-ranging pigs that were suspected to have had contact with warthogs, including carcasses left lying in the fields. Subtypes 1a and 1b of ASFV were identified in *O. (O.) compactus* ticks collected from burrows on property NCP02 (Figures 4.1; 4.3; Table 4.1) where subtype 1a had caused an outbreak of disease in 2017 in penned pigs fed fresh entrails of a warthog shot on the same property. While the infectivity of warthog offal for domestic pigs is disputed, low doses of virus were shown to be infective for pigs by mouth [133] and transmission of virus by the feeding of warthog entrails on property NCP02 tends to confirm this observation. For biosafety and legal reasons only government veterinary officials are permitted entry to premises under quarantine following diagnosis of ASF outbreaks, so it was not possible to access a farm in North West Province only 10 km south of the controlled area where domestic pigs and Eurasian wild boars that escaped from their pens succumbed to the disease in 2019 after suspected contact with warthogs (Figure 4.1), but we detected ASFV nucleic acid in 7/39 pools of *O. (O.) moubata* s.s. ticks collected on two neighbouring properties; locations NWP04 and NWP05 (Figure 4.3; Table 4.1). The virus subtype detected, 1b, corresponds to that recovered in the disease outbreak. Since warthogs are not contagious for each other or domestic pigs [134,135], reference to transmission by contact with warthogs above is meant to imply consumption of infected warthog tissues or transmission through the intermediary of ticks. Thus, there is strong evidence that virus in sylvatic circulation outside the controlled area has ignited infection in domestic pigs on occasion. However, no indication of the involvement of warthogs or ticks was obtained in the extensive series of outbreaks of disease that occurred in pigs in southern Gauteng Province and adjacent south-western Mpumalanga Province in 2016-2020 [12,13,136].

In 2018 there was an outbreak of disease associated with ASFV subtype 1a on a property in western Northern Cape Province (Figures 4.1; 4.5) in pigs shortly after they had been introduced from a farm in the Kimberley area that remained free of infection [6-9]. Accordingly, it was intended to extend the current investigations to the western part of Northern Cape Province and the adjacent Western Cape Province to seek further possible instances of unrecorded presence of warthogs and potentially infected ticks, or simply to explore prevalence and distribution of *Ornithodoros* species and virus irrespective of the presence of

suids, but this was precluded by COVID-19 travel restrictions. Likewise, it was intended to extend observations to the eastern Free State Province and the adjacent KwaZulu-Natal Province to determine how far northwards the distribution of *O. (P.) zumpti* extends from Eastern Cape Province and how far west and south the distribution of *O. (O.) phacochoerus* extends (Figure 4.6), but this was also precluded by travel restrictions.

Since the present study was intended merely to seek evidence of circulation of ASFV in warthogs and ticks beyond the controlled area in South Africa, definitive analysis of the mechanisms of spread of the sylvatic cycle is not possible. Nevertheless, it is clear that opportunities for spread of infection include translocation of live warthogs and transportation of carcasses, as well as informal trade in potentially infected pigs and pork products [137,138]. In fact, the genotype IV ASFV isolate with GenBank accession number AF449477 in Figure 4.4, was obtained from a warthog carcass confiscated at a roadblock in 1999 while being transported without a veterinary permit (L. Heath, unpublished).

Despite the evidence of widespread sylvatic circulation of ASFV beyond the controlled area obtained in the present project, and the fact that multiple types of the virus are involved, there was no indication of dispersal and establishment of tick populations beyond their expected species distribution ranges [21]. Thus, a high prevalence of ASFV infection was detected in *O. compactus* within its historic distribution range, but no other tick species was encountered during the admittedly limited sampling of burrows in this area. Even if historically there had been circulation of ASFV between *O. compactus* and the Cape warthog (*Phacochoerus aethiopicus aethiopicus*) that became extinct in 1896 [139], it is unlikely that the virus would have been maintained solely by transovarial transmission in ticks in the absence of viraemic hosts until introductions of the common warthog commenced more than six decades later [15,52].

Unlike ixodids, larval ticks of the subgenus *Ornithodoros* tend to moult directly into the first nymphal instar without feeding and nymphs undergo 2-8 moults; the number varies with species and may be reduced where early nymphal instars obtain inadequate blood meals. Furthermore, argasid ticks including *Ornithodoros* species do not generally attach to their hosts to feed over a period of days to weeks, but engorge rapidly, within minutes to hours, while their hosts are at rest and then detach to moult to the succeeding instar over a period of weeks to months, or to lay eggs as adult females [43,45]. However, ticks that have not completed feeding may be passively conveyed out of burrows when the hosts leave in the mornings, and 46/129 (35.7%) warthogs shot at monthly intervals over the course of a year at three sites in South Africa and Namibia were found to carry a total of 616 *Ornithodoros* nymphs

and 2 adults [140-142]. It can be surmised that nymphs more readily become enmeshed and cling to the coats of warthogs while the heavier adult ticks have a greater tendency to fall off.

Warthogs are non-migratory and non-territorial, so that matriarchal family groups may have overlapping home ranges of approximately 20-170 hectare in different parts of southern Africa. They adapt disused burrows of antbears and certain other animals such as porcupines, or culverts and erosion gullies, for use as shelters at night. They display some tenure of burrows but change occupancy frequently, utilizing up to 10 different shelters [143]. At least 26 other species of mainly small vertebrates have been recorded as utilizing antbear burrows in South Africa [144], so that regular traffic of warthogs and other animals between burrows within relatively short distances of each other could account for localized spread of ticks and virus. However, calculations made for long distance dispersal of immature *Ornithodoros* ticks parasitic on sea birds indicate that the probability is very small that dispersed ticks would successfully give rise to adult male and female ticks capable of breeding at remote locations [145]. The probability that breeding adult tick vectors infected by a pathogen could successfully be established at a remote site is even smaller; there is a greater chance of a translocated tick transmitting infection than there is of establishing a viable breeding colony of ticks. This model seems to fit the pattern of tick infection observed in the present study. It is planned to conduct vector competence tests on selected tick species and follow-up field studies at provincial level to determine the full extent and mechanisms of spread of sylvatic circulation of ASFV in South Africa.

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4.7 Competing interests

The authors declare that no financial or personal relationships have inappropriately influenced them in preparing this manuscript.

4.8 Ethics statement

Research ethics approval was obtained from the Animal Ethics Committee, UP, and the Research Committee of the Faculty of Veterinary Science, UP. Section 20 approval in terms of the Animal Diseases Act 35 of 1984 was obtained from the Director of Animal Health, Department of Agriculture, Land Reform and Rural Development, South Africa. Research agreements were concluded with the Scientific Services Committee of the South African National Parks Board, and written informed consent was obtained from the owners of other properties from which samples were taken. Samples were transported in appropriate biosafety packaging under permit from relevant State Veterinarians and laboratory procedures were performed under Animal BSL3 conditions in TADL. Samples requiring threatened or protected species permits (ToPS) are covered by standing Faculty of Veterinary Science, University of Pretoria permit.

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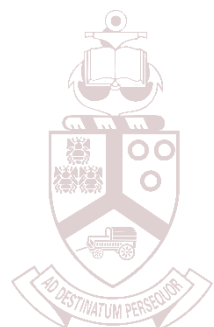
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CHAPTER 5

Concluding remarks and implications



5.1 Concluding remarks and implications

The findings of the present study were duly presented as three published manuscripts (Craig et al., 2021a; 2021b; 2022). The first publication (Craig et al., 2021a; Chapter 2), reported new host and distribution records for the tick *O. (P.) zumpti*, including finding of the tick in warthog burrows for the first time, and was intended to stimulate interest in the investigation of the possible involvement of the tick in the ongoing ASF outbreaks in the Eastern Cape Province. Furthermore, the results of Craig et al., (2021a) suggest high levels of intra-specific-variation within a monophyletic *O. zumpti* lineage, with the constituent taxa having >95% sequence identity across the 16S rRNA gene region. Further studies including full mitochondrial sequencing and examination of additional samples from the field are being pursued at the ARC-OVR Gertrude Theiler Tick Museum.

Vector competence studies with *O. (P.) zumpti* and ASFV are scheduled to take place at TADL. Initially it was hoped to include the establishment of *Ornithodoros* tick colonies at TADL in the present project to facilitate vector competence studies also with *O. (O.) compactus* and some of the recently described new *Ornithodoros* species, but the facilities were ruled unsuitable for the housing of tick colonies. In any event, it can be surmised from partial 16S rRNA sequence data that some of the newly described species correspond to known vectors previously included erroneously in *O. (O.) porcinus* as indicated by Bastos et al. (2009).

Both the detection of antibody in warthogs and virus in ticks (Craig et al., 2021b; 2022; Chapters 3 and 4) indicate that there is need for more extensive surveillance of the distribution of sylvatic circulation of ASFV outside the controlled area, including the potential existence of warthogs, ticks and virus in areas not covered by the present study. The discovery of new *Ornithodoros* species is considered a possibility as geographical sampling is extended, as suggested by Bakkes et al., (2018). The mechanisms of tick infection and transmission of ASFV remain to be fully understood and appear to be regulated by multiple tick species- and virus strain-specific factors (Pereira de Olivera et al., 2019; Craig et al., 2022). Since it is clear that ASFV infection in confirmed vectors represents a co-evolved biological system (Kleiboeker et al., 1998), it remains uncertain whether the latent ability to transmit the virus is a general property of *Ornithodoros* ticks. As ASF continues to spread in South Africa, the present study highlights the usefulness of exploring the diversity of soft ticks together with investigating the ability of the ticks to become infected and transmit infection to susceptible animals. The study also confirms the advantage of using extralimital warthogs in determining the disease status of an area, as if present, ASF seropositivity usually has a high prevalence.

The incidental receipt of blood samples from Eurasian wild boars encountered in North West, Gauteng and Free State Provinces (Craig et al., 2021b) is cause for concern given that the species has proved to be a highly invasive elsewhere in the world. In the course of the project, the investigator became aware that wild boars are widely advertised in local print media and online for sale or hunting. Similarly, warthog meat or butchered carcasses are widely advertised for sale in South Africa.

The detection of genotype III virus in western Free State Province (Craig et al., 2022) raises the question of the possible occurrence of other small foci of infection with unexpected genotypes.

Finally, the potential role of alternative hosts of *Ornithodoros* ticks in incidental spread of tick and virus warrants further investigation.

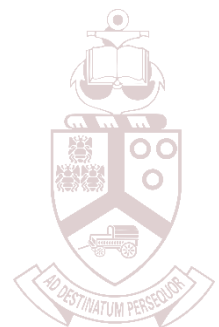
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Pereira de Oliveira, R., Hutet, E., Paboeuf, F., Duhayon, M., Boinas, F., Perez de Leon, A., Filatov, S., Vial, L., Le Potier, M-F. (2019). Comparative vector competence of the Afrotropical soft tick *Ornithodoros moubata* and Palearctic species, *O. erraticus* and *O. verrucosus*, for African swine fever virus strains circulating in Eurasia. *PLoS ONE* 14(11): e0225657

APPENDICES



APPENDIX A – RESEARCH ETHICS APPROVAL



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Research Ethics Committee

PROJECT TITLE	Interrelationships of Warthogs, <i>Ornithodoros</i> ticks and African swine fever virus in South Africa.
PROJECT NUMBER	REC081-18
RESEARCHER/PRINCIPAL INVESTIGATOR	Anthony Craig

DISSERTATION/THESIS SUBMITTED FOR	PhD
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SUPERVISOR	Robert Swanepoel
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APPROVED	Date 08 November 2018
CHAIRMAN: UP Research Ethics Committee	Signature <i>A.M. Duman</i>

APPENDIX B – ANIMAL ETHICS APPROVAL



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	Interrelationships of Warthogs, <i>Ornithodoros porcinus</i> ticks and African Swine Fever virus in South Africa
PROJECT NUMBER	V011-18
RESEARCHER/PRINCIPAL INVESTIGATOR	Mr AF Craig

STUDENT NUMBER (where applicable)	U_24282449
DISSERTATION/THESIS SUBMITTED FOR	PhD

ANIMAL SPECIES	Warthogs
NUMBER OF SAMPLES	Number of blood samples to be provided
Approval period to use animals for research/testing purposes	March 2018 – March 2019
SUPERVISOR	Prof. R Swanepoel

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED (*With condition)	Date 4 April 2018
CHAIRMAN: UP Animal Ethics Committee	Signature 

CONDITION: Tissue can only be sourced from hunters registered with the hunting association. While the process of hunting is not ethical questionable, the hunting by inexperienced persons can results in welfare issues. The hunting associations have properly codes of conduct to prevent welfare issues, such as the experience of the hunter and a willingness to always track down injured animals for euthanasia, irrespective how difficult it may be.

S4285-15

APPENDIX C – DALRRD SECTION 20 RESEARCH APPROVALS



agriculture, forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6

Anthony Francis Craig
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Onderstepoort
0110
Email: bob.swanepoel@up.ac.za & af.craig83@gmail.com

Dear Dr Craig,

RE: CONSOLIDATED PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your previous Section 20 permission and amendments granted, dated 2018-10-25, 2019-02-18 and 2019-03-08, and subsequent correspondence refers. This consolidated permission for this project replaces the previous permission granted and expands on it with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form 2018-08-01 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. All potentially infectious material utilised, collected or generated during the study are to be destroyed at the completion of the study. Records must be kept for five years for auditing purposes;

Blood samples on filter paper:

4. Blood samples from warthog/bushpig may be collected on filter paper by either the researchers or hunters/landowners. These dried filter paper strips must be placed in sealed envelopes, which is placed in biological sample ziplock bags;

5. Samples collected by hunters/landowners may only be collected from legally hunted warthogs/bushpigs, for which a permit was issued by the SAHGCA, and can be transported to the SAHGCA headquarters, from which it will be moved to the TAD laboratory for testing;

Blood, serum and tissue samples:

6. Fresh samples from hunted warthog/bushpig/European wild boar or feral pigs may be collected by the researchers only, subject to any conditions stipulated by the responsible state veterinarian;
7. Fresh samples may only be collected from legally hunted wild pigs, where permission has been obtained from the owners or authority and require a red cross permit, issued by the responsible state veterinarian and can only be transported directly to the TAD laboratory for testing;

Ornithodoros tick samples:

8. *Ornithodoros* ticks may only be collected where permission has been granted by the local state veterinarian responsible for the area;
9. *Ornithodoros* ticks must be transported in the triple biocontainment packaging as was described in the application correspondence and may only be moved with the required movement permit from the State Veterinary office responsible for the area;
10. All *Ornithodoros* ticks collected may only be transported directly to TAD laboratory for testing;
11. At the completion of the PCR testing of the *Ornithodoros* ticks, the ticks must be destroyed and incinerated;

Movement of samples out of TAD:

12. DNA extractions are to be done in the BSL 3 facility at TAD, ARC-OVR and only inactivated DNA samples may be sent to the Department of Veterinary Tropical Diseases at the University of Pretoria as per the TAD SOP on removal of samples from the BSL 3 laboratory;
13. Samples fixed in 10% formalin may be moved from TAD to the Section of Pathology, Faculty of Veterinary Science following disinfection of the outside of the container;

Storage of samples:

14. Should any virus be isolated at TADL, these can be stored in the BSL 3 TAD laboratory, any further use of the virus would be subject to a new Section 20 approval;

General:

15. No diagnostic results, based on the PCR performed at DVTD may be issued and results may only be used for research purposes, however, any suspicions of ASF must be reported to the local state veterinarian and DAFF;
16. All samples must be packaged and transported in accordance with International Air Transport Association (IATA) requirements and the National Road Traffic Act, 1996 (Act No. 93 of 1996);

17. All waste must be disposed of as biohazardous waste by a registered waste contractor;
18. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act 19 of 1982);
19. Any further use or distribution of samples collected for this study is subject to obtaining a separate Section 20 approval;
20. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Interrelationships of warthogs, *Ornithodoros* ticks and African swine fever virus in South Africa

Researcher: Dr Anthony Francis Craig

Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

Our ref Number: 12/11/1/1/6

Your ref: VO11-18

Expiry date: 2021-03

Kind regards,



DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2019-08-08

- 3 -

SUBJECT: S20 APPROVAL FOR: INTERRELATIONSHIPS OF WARTHOGS, *ORNITHODOROS* TICKS AND AFRICAN SWINE FEVER VIRUS IN SOUTH AFRICA - LJVR



agriculture, forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za

Reference: 12/11/1/1/6 (1057)

Dr Anthony Francis Craig
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Tel: 012 529 8566
E-mail: bob.swanepoel@up.ac.za

Dear Dr Craig,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your application sent with the email on 20 February 2019 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form received on 20 February 2019 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under this Section 20 permit. Please apply in writing to HerryG@daff.gov.za;
3. Only the specified warthog (serum and lymphnode) and hyena (serum) samples, for which permission for removal from the SANParks Veterinary Wildlife Services Biobank has been obtained, may be utilised for the purposes of this study;
4. The specified samples (aliquots) may only be sent to the Transboundary Animal Diseases Programme Laboratory (TADPL) under cover of a Red Cross Permit for

testing. Samples must be packaged and transported in accordance the National Road Traffic Act, 1996 (Act No. 93 of 1996);

5. African Swine Fever PCR and ELISA testing on the specified samples may only be conducted at TADPL;
6. For the importation of any required diagnostic kits or materials, a valid Veterinary Import Permit must be obtained and all conditions stipulated therein adhered to. Records must be kept for five years for auditing purposes;
7. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study. Records must be kept for five years for auditing purposes;
8. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: *"The testing of warthog serum and tissue samples in the Sanparks Kruger National Park Biobank for African swine fever virus DNA and antibody"*

Researcher: Dr Anthony Francis Craig

Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

Our ref Number: 12/11/1/1/6 (1057)

Your ref: VO11-18

Expiry date: December 2019

Kind regards,



DR. MPHOMAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2019-05-22

- 2 -

SUBJECT: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)



**agriculture, land reform
& rural development**

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform & Rural Development
Private Bag X158, Pretoria 0001

Enquiries: Mr Herry Goldo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/8 (1733 LJvR)

Anthony Francis Craig
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Onderstepoort
0110
Email: bob.swanepoel@up.ac.za & af.craig83@gmail.com

Dear Dr Craig,

**RE: AMENDMENT TO PERMISSION TO DO RESEARCH IN TERMS OF SECTION
20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)**

Your request for amendment and extension, sent per email on 9 November 2020, on the consolidated Section 20 permission previously granted (dated 2019-08-08), refers. I am pleased to inform you that permission is hereby granted for the amendments to the study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The study is approved as per the application form sent 9 November 2020 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under the Section 20 permit and amendment letters. Please apply in writing to HerryG@daff.gov.za;
3. Dried blood samples on filter paper strips from road-kills can be collected by Skukuza state veterinary officials and Northern Cape state veterinary officials

and sent to the Transboundary Animal Diseases laboratory for testing for antibodies against ASFV and/or PCR testing for ASFV nucleic acid;

4. Any proposed publication emanating from this research should be sent for comment to the Director: Animal Health prior to submission for publication;
5. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Interrelationships of warthogs, *Ornithodoros* ticks and African swine fever virus in South Africa

Researcher: Dr Anthony Francis Craig

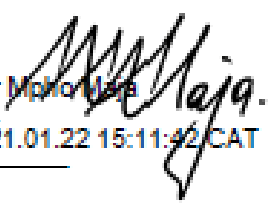
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

Our ref Number: 12/11/1/1/6 (1733)

Your ref: VO11-18

Expiry date: 2022-12

Kind regards,

Name: Dr Mpho Maja
Reason: 
Date: 2021.01.22 15:11:42 CAT

DR. MPHO MAJA

DIRECTOR OF ANIMAL HEALTH

Date:

- 2 -

SUBJECT: S20 AMENDMENT FOR: INTERRELATIONSHIPS OF WARTHOGS, *ORNITHODOROS* TICKS AND AFRICAN SWINE FEVER VIRUS IN SOUTH AFRICA - LJV



agriculture, forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za

Reference: 12/11/1/1/6

Anthony Francis Craig
Department of Veterinary Tropical Diseases
Faculty of Veterinary Science
University of Pretoria
Onderstepoort
0110
Email: bob.swanepoel@up.ac.za & af.craig83@gmail.com

Dear Dr Craig,

RE: AMENDMENT TO PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984)

Your request for amendment, sent per email on 7 October 2019, on the consolidated Section 20 permission previously granted (dated 2019-08-08), refers. I am pleased to inform you that permission is hereby granted for the amendments to the study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this study under the Section 20 permit and amendment letters. Please apply in writing to HerryG@daff.gov.za;
3. Ornithodorid ticks (tampans) from the field collection may only be removed from the Transboundary Animal Diseases laboratory if the ticks are fixed in

formalin and the external surface of the specimen containers have been disinfected as per the TAD SOP on removal of samples from the BSL 3 laboratory;

4. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 approval.

Title of research/study: Interrelationships of warthogs, *Ornithodoros* ticks and African swine fever virus in South Africa

Researcher: Dr Anthony Francis Craig

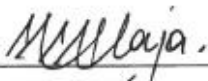
Institution: Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria

Our ref Number: 12/11/1/1/6

Your ref: VO11-18

Expiry date: 2021-03

Kind regards,



DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH
Date: 2019-10-22

- 2 -

SUBJECT: S20 AMENDMENT FOR: INTERRELATIONSHIPS OF WARTHOGS, *ORNITHODOROS* TICKS AND AFRICAN SWINE FEVER VIRUS IN SOUTH AFRICA - LJVR



agriculture, forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Forestry and Fisheries
Private Bag X138, Pretoria 0001

Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@daff.gov.za
Reference: 12/11/1/1/6

Anthony Francis Craig
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Onderstepoort
Tel: (012) 529 8566
E-mail: bob.swanepoel@up.ac.za ; af.craig83@gmail.com

RE: AMENDMENT OF SECTION 20 APPROVAL IN TERMS OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984) FOR: "INTERRELATIONSHIPS OF WARTHOGS, ORNITHODOROS TICKS AND AFRICAN SWINE FEVER VIRUS IN SOUTH AFRICA"

An amendment is hereby granted on the Section 20 approval that was issued for the above mentioned study on 8 August 2019 (attached):

- i) The 23 serum samples obtained from warthogs during a culling operation in Mokala National Park (2017), which is currently stored in the Kimberley branch of the SANParks VWS Biobank, may be utilised as part of this study;
- ii) These specified samples (aliquots) may only be sent to the Transboundary Animal Diseases Programme Laboratory (TADPL) under the cover of a Red Cross Permit for African swine fever testing. Samples must be packaged and transported in accordance the National Road Traffic Act, 1996 (Act No. 93 of 1996);
- iii) All other conditions as specified in the Section 20 approval of 8 August 2019 remain in full effect.

Kind regards,

DR. MPHO MAJA
DIRECTOR OF ANIMAL HEALTH

Date: 2020-03-19

APPENDIX D – CONSENT TEMPLATE



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

Research project: Interrelationships of warthogs, *Ornithodoros* ticks and African swine fever virus in South Africa

Consent Form

I, ... (Owner/Representative) of (Property), agree that the samples indicated below may be obtained from the above property during the sampling period 2019-2020 (please tick appropriate box/es):

- ☐ Blood samples collected on filter-paper strips from hunted or culled warthogs and/or bush pigs to be tested for genetic identification of the animal species and detection of evidence of infection with African swine fever virus.
- ☐ *Ornithodoros* ticks (tampans) collected from warthog burrows and/or sandy habitats for the identification of tampan species, detection of virus content, and identification of animal type from which the tampan took its blood meal (many kinds of animal visit warthog burrows).

This research is being conducted in collaboration between the Department of Veterinary Tropical Diseases, University of Pretoria and the Onderstepoort Veterinary Research - Transboundary Animal Disease Programme Laboratory.

The undersigned parties further agree that all research-associated costs will be covered by the researcher(s), and no compensation will be payable to the property's owner or anybody else.

The undersigned parties further agree that this form serves to fully indemnify the University of Pretoria and the undersigned researcher(s) against any future claims resulting from any specified procedure by or on behalf of the property owner.

The undersigned parties further agree that no material of any kind, including data and research findings, obtained or resulting from the project, would be passed on to any third party or used for any purpose other than that specified in this form, except with the written consent of the undersigned owner of the property.

Findings will be tabulated by region and presented without reference to owners or properties in a postgraduate thesis and scientific publications.

As owner/representative it is my right to withdraw my information and samples from the project.

.....
Owner/representative

.....
Researcher/s

.....
Witness

.....
Date

Afrikaans op keersy

Navorsingsprojek: Interaksie tussen vlakvarke, *Ornithodoros* bosluise en Afrikaanse varkpesvirus in Suid Afrika

Toestemmings vorm

Ek, ... (eienaar/verteenwoordiger) van (eiendom), gee toestemming dat die monsters soos aangedui hieronder, van die bogenoemde eiendom, gedurende die tydperk 2019-2020 bekom mag word (merk asb. die toepaslike opsies):

- ☐ Bloedmonsters versamel op filtreerpapier-strokies van vlakvarke of bosvarke wat gejag was, om tertoets vir genetiese identifikasie van die spesies en tekens van besmetting met Afrikaansevarkpesvirus.
- ☐ *Ornithodoros* bosluise (tampanne) versamel uit vlakvarkgate of sanderige gebiede vir dieidentifisering van tampan spesies, bepaling of die tampan besmet is, en identifisering van diediersoort waaruit die tampan se bloed-maal gesuig is (baie diersoorte besoek vlakvarkgate).

Hierdie navorsing is 'n samewerking tussen die Universiteit van Pretoria se Departement van Veterinêre Tropiese Siektes en die Onderstepoort Veterinary Research - Transboundary Animal Disease Programme Laboratory.

Die ondergetekende partye kom ooreen dat alle navorsings-geassosieerde kostes deur die navorsers gedra sal word en dat geen vergoeding aan die eienaar van die landgoed of enige iemand anders betaal sal word nie.

Die ondergetekende partye kom verder ooreen dat hierdie dokument dien om die Universiteit van Pretoria asook die navorsers kwyt te skel van enige toekomstige eise wat mag voortspruit uit enige prosedure deur of namens die eienaar van die landgoed.

Die ondergetekende partye kom verder ooreen dat geen materiaal van watter aard ook al, insluitende data en bevindings van die projek, of wat voort spruit uit die projek, na enige derdeparty oorgedra sal word nie en dat dit ook nie vir enige ander doel as wat in hierdie ooreenkoms gestipuleer word, gebruik sal word nie, behalwe met skriftelike toestemming van die ondergetekende eienaar van die landgoed.

Bevindings sal per streek gerapporteer word, sonder om te verwys na die eienaar of die landgoed, in 'n nagraadse tesis en wetenskaplike publikasies.

As eienaar/verteenwoordiger is dit my reg om my inligting en monsters van die projek te onttrek.

.....
Handtekening eienaar/verteenwoordiger

.....
Handtekening navorser/s

.....
Handtekening getuie

.....
Datum

English on reverse side

APPENDIX E – PROJECT SUMMARY PREPARED FOR THE SOUTH AFRICAN HUNTERS AND GAME CONSERVATION ASSOCIATION



UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science

RESEARCH PROJECT ON AFRICAN SWINE FEVER

If warthogs and/or bush pigs are hunted on your property, we need your assistance in a research project being conducted in the Faculty of Veterinary Science, University of Pretoria.

WHAT DOES THE RESEARCH PROJECT INVOLVE?

Our intention is to perform a survey for evidence of African swine fever (ASF) virus infection on dried blood samples collected for us on filter paper strips from hunted warthogs or bushpigs on properties that agree to participate. The survey should cover the whole the country, but only a few animals (1 to 5) need to be sampled per property. In addition, the research team would need to collect tampons from burrows on selected properties.

Laboratory tests will be conducted at the ARC-Onderstepoort Veterinary Research Transboundary Animal Disease Laboratory. Findings will be tabulated by region and presented without reference to owners or properties in a postgraduate thesis and scientific publications.

WHAT IS AFRICAN SWINE FEVER?

ASF is a fatal disease of domestic pigs, caused by a virus that is carried by *Ornithodoros* ticks (also called tampons) and warthogs which do not become sick. Humans are not affected.

Tampons do not attach like ordinary ticks, but feed rapidly while warthogs are at rest in burrows. However, they can be carried short distances on warthogs to the vicinity of piggeries where they can transmit infection to pigs.

Warthogs themselves are not infectious for pigs unless they are killed, and their meat and organs are fed to pigs in swill. Bush pigs are also resistant to ASF, but their role as a source of infection for domestic pigs is uncertain.

HOW IS THE DISEASE CONTROLLED?

An 'ASF control zone' was declared in 1935 to include virtually all of Limpopo Province plus adjacent parts of Mpumalanga and North West Provinces, and part of northern KwaZulu-Natal Province where warthogs were known to occur.

Regulations require that domestic pigs farmed commercially within the control zone should be kept in double-fenced piggeries to exclude contact with warthogs, and have to be butchered at designated abattoirs.

Warthogs and bush pigs may only be removed from properties anywhere in the country under veterinary permit, and carcasses should have the skin, head, hooves and entrails with lymph glands removed.

The control measures proved effective for many years, with only a few outbreaks occurring outside of the control zone due to the illegal movement of infected pigs from Limpopo Province.

WHY IS NEW RESEARCH ON ASF CONSIDERED NECESSARY?

In recent outbreaks of ASF in domestic pigs in North West, Free State and Northern Cape Provinces in 2016-2018, no conclusive evidence of the source of the infection was found.

There have been large-scale translocations of warthogs over the past 50 years, so that they are now much more widely distributed in the country than when the control zone was declared in 1935.

New species of tampons have been discovered, so there is some uncertainty about the distribution and ability of the various species to transmit ASF virus.

Since the disease can be devastating for the pig industry, the purpose of the project is to determine whether there is evidence of circulation of ASF virus beyond the control zone. The existing regulations already apply to warthogs and bush pigs throughout the country, so the information is required to ensure that protective measures for the pig farming industry are appropriate.



NAVORSING OP AFRIKAANSE VARKPES

Indien vlakvarke en/of bosvarke op u eiendom gejaag word, word u samewerking verlang in 'n navorsing projek deur die Fakulteit Veeartsenykunde by die Universiteit van Pretoria

WAT BEHELS DIE NAVORSING?

Die doel is om 'n opname uit te voer vir bewyse van Afrikaanse varkpes (AVP) virus in droë bloedmonsters wat op filtreerpapier strokies namens ons gekollekteer word van vlakvarke en bosvarke op plase wat bereid is om deel te neem. Die opname is bedoel om die hele land te dek, maar monsters van selfs 1 tot 5 diere word benodig per eiendom. Daarby sou die navorsers tampan bosluise wou versamel van vlakvark gate op uitgesoekte plase. Laboratoriums toetse sal by die ARC-Onderstepoort Veterinary Research Transboundary Animal Disease Laboratory uitgevoer word. Bevindings sal volgens streek, met geen verwysing na eienaars of eiendom, in 'n nagraadse tesis en wetenskaplike publikasies weergegee word.

WAT IS AFRIKAANSE VARKPES?

Afrikaanse varkpes (AVP) is 'n dodelike siekte van varke, wat veroorsaak word deur 'n virus wat tussen tampan bosluise en vlakvarke sirkuleer. Vlakvarke word nie siek van die virus nie. Mense word nie aangetas nie.

Tampans heg nie vas soos gewone bosluise nie maar voed vinnig wanneer die vlakvarke in hulle gate verkeer. Hulle kan egter kort afstande op vlakvarke gedra word en sodoende in kontak kom met varke wat dan besmet kan word.

Vlakvarke kan self nie die infeksie aan varke oordra nie, behalwe as hulle van kant gemaak word en hulle vleis en organe in afval aan varke gevoer word. Bosvarke word ook nie siek nie, maar hulle rol as bron van besmetting vir varke is tans onseker.

HOE WORD DIE SIEKTE BEHEER?

'n AVP beheer-sone was in 1935 verklaar en sluit omtrent die hele Limpopo provinsie asook aangrensende dele van Mpumalanga, Noordwes en noordelike Kwazulu-Natal in, waar die voorkoms van vlakvarke destyds bekend was.

Regulasies vereis dat varke in 'n kommersiële varkboerdery in die beheer-sone agter dubbel omheinings aangehou moet word om sodoende kontak met vlakvarke te beperk. Die varke mag slegs by aangewese abattoirs geslag word.

Vlakvarke en bosvarke mag slegs onder veteriniere permit van enige eiendom in Suid-Afrika verwyder word en die vel, pote, kop, en binnegoed met limfknope moet ook van alle karkasse verwyder word.

Die beheermaatreëls was vir baie jare effektief met slegs enkele uitbrekings wat buite die beheer-sone voorgekom het as gevolg van die onwettige vervoer van besmette varke vanuit Limpopo.

WAAROM IS NUWE NAVORSING OP AVP NODIG?

In onlangse uitbrekings van AVP in varke in Noordwes, Vrystaat en Noordkaap gedurende 2016-2018 kon geen oortuigende bewyse van die bron van besmetting gevind word nie.

Daar het grootskaalse verskuiwing van vlakvarke gedurende die afgelope 50 jaar plaasgevind en vlakvarke kom nou baie meer wyd verspreid in die Land voor as toe die beheer-sone in 1935 verklaar was.

Nuwe spesies tampan bosluise is onlangs beskryf, derhalwe bestaan daar onsekerheid oor die huidige verspreiding en vermoë van die verskillende spesies om AVP oor te dra.

Aangesien die siekte 'n vernietigende effek op die vark industrie kan hê, is die doel van die projek om vas te stel of daar bewyse is dat die AVP virus buite die beheer-sone sirkuleer. Die bestaande regulasies is al reeds landswyd op vlakvarke en bosvarke van toepassing, en die inligting word dus benodig vir beter insae in die beskermende maatreëls vir die vark boerderynyweheid.

APPENDIX F – BLOOD COLLECTION INSTRUCTIONS PREPARED FOR THE SOUTH AFRICAN HUNTERS AND GAME CONSERVATION ASSOCIATION



AFRICAN SWINE FEVER RESEARCH PROJECT: INSTRUCTIONS FOR COLLECTION OF BLOOD SAMPLES ON FILTER PAPER STRIPS

- Blood samples for laboratory tests are collected on Nobuto filter paper strips.
- Participants are supplied with 4 brown peel-n-seal envelopes, each containing 4 Nobuto filter paper strips (1 envelope/4 filter paper strips per animal).
- Collect 4 samples from each warthog or bushpig by applying the narrow end of the filter paper strip to blood oozing from bullet wounds, allowing blood to soak up the entire narrow section of the strip (Fig. 1).



Figure 1: Absorbing blood with Nobuto filter paper strip

- Dry the strip by waving in the air or propping up on a clean surface for a few minutes to dry.
- Fill in the details of the sample on the envelope provided, place the dry strips in the envelope and seal (Fig. 2).

A brown paper envelope with a white label. The label contains the following text: 'Permit No. _____', 'Animal _____', 'Date collected _____', 'Sex M F (please circle) _____', 'Age (young, adult, etc.) _____', 'Collected by _____', 'Location/GPS _____', and '*Store in a cool place*'.

Permit No. _____
Animal _____
Date collected _____
Sex M F (please circle) _____
Age (young, adult, etc.) _____
Collected by _____
Location/GPS _____
Store in a cool place

Figure 2: Envelope for storage of blood filled strips



Figure 3: Biological sample Ziplock bag

- Place sealed envelopes in the double layer Biological Sample Ziplock bags supplied together with a duly completed and signed consent form and store in a cool place until collected by researcher (Fig. 3).

Dried samples are stable for a month if stored away from sunlight.

APPENDIX G – MATERIAL TRANSFER AGREEMENT (SANPARKS)

Reference: BMTA 002/19



MATERIAL TRANSFER AGREEMENT

BETWEEN

SOUTH AFRICAN NATIONAL PARKS

herein represented by **Dr Peter Buss**

in his/her capacity as **Veterinary Senior Manager: VWS**

(hereinafter referred to as "SANParks")

AND

[Department of Veterinary Tropical Diseases]

herein represented by **Prof R Swanepoel**

in his/her capacity as **Researcher**

(hereinafter referred to as "Recipient")

WHEREAS the Recipient has requested the Material from SANParks;

AND WHEREAS SANParks accepted the Recipients' request for the Material subject to the terms and conditions as stipulated hereunder:

APPENDIX H – CONSOLIDATED TICK RESULTS

Tick pool	Code	Province ¹	QGDC ²	No. of Ticks	Instar ³	Cq Mean	p72 qPCR	p72 PCR	Genbank # p72	CVR PCR	Genbank # CVR	16S rRNA PCR	Genbank # 16S rRNA	Cyt b PCR
TP001	GKNP-02	LP	E031S022CA	5	N3/4	0,00	neg							
TP002	GKNP-02	LP	E031S022CA	5	N3/4	0,00	neg							
TP003	GKNP-02	LP	E031S022CA	5	N3/4	0,00	neg							
TP004	GKNP-02	LP	E031S022CA	5	N3/4	0,00	neg							
TP005	GKNP-01	LP	E031S024AC	3	N4/Ad	0,00	neg					POS	OK136965	
TP006	GKNP-01	LP	E031S024AC	3	N4/Ad	0,00	neg					POS	OK136966	
TP007	GKNP-01	LP	E031S024AC	3	N4/Ad	0,00	neg					POS	OK136967	
TP008	GKNP-01	LP	E031S024AC	3	N4/Ad	0,00	neg					POS	OK136968	
TP009	GKNP-01	LP	E031S024AC	3	N4/Ad	0,00	neg					POS	OK136969	
TP010	GKNP-01	LP	E031S024AC	5	N2/3	0,00	neg					POS	OK136970	
TP011	GKNP-01	LP	E031S024AC	10	N2	0,00	neg					POS	OK136971	
TP012	GKNP-01	LP	E031S024AC	15	N1/2	0,00	neg					POS	OK136973	POS
TP013	GKNP-01	LP	E031S024AC	6	N2	21,67	POS	POS	OK148637			POS	OK136974	
TP014	GKNP-01	LP	E031S024AC	5	N2/3	0,00	neg					POS	OK136975	
TP015	GKNP-01	LP	E031S024AC	10	N2	0,00	neg					POS	OK136977	
TP016	GKNP-01	LP	E031S024AC	10	N2	0,00	neg					POS	OK136978	

TP017	GKNP-03	LP	E031S022CA	2	Ad	0,00	neg	POS	OK136979	
TP018	GKNP-03	LP	E031S022CA	2	Ad	0,00	neg			neg
TP019	GKNP-03	LP	E031S022CA	2	Ad	0,00	neg	POS	OK136983	
TP020	GKNP-03	LP	E031S022CA	4	N1/4	0,00	neg			
TP021	GKNP-02	LP	E031S022CA	5	N3	0,00	neg			
TP022	GKNP-02	LP	E031S022CA	5	N3	0,00	neg			
TP023	GKNP-02	LP	E031S022CA	5	N3	0,00	neg			
TP024	GKNP-02	LP	E031S022CA	5	N3	0,00	neg	POS	OK136994	
TP025	GKNP-02	LP	E031S022CA	15	N1	0,00	neg			neg
TP026	GKNP-02	LP	E031S022CA	15	N1	0,00	neg	POS	OK137000	
TP027	GKNP-02	LP	E031S022CA	2	Ad	0,00	neg			
TP028	GKNP-02	LP	E031S022CA	2	Ad	0,00	neg			
TP029	GKNP-02	LP	E031S022CA	2	Ad	0,00	neg			
TP030	GKNP-02	LP	E031S022CA	2	Ad	0,00	neg			
TP031	GKNP-02	LP	E031S022CA	2	Ad	0,00	neg			
TP032	GKNP-02	LP	E031S022CA	2	Ad	0,00	neg			
TP033	GKNP-02	LP	E031S022CA	2	Ad	0,00	neg			
TP034	GKNP-02	LP	E031S022CA	5	N3	0,00	neg			
TP035	GKNP-02	LP	E031S022CA	5	N3	0,00	neg			

TP036	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP037	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP038	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP039	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP040	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP041	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP042	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP043	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP044	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP045	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP046	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP047	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP048	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP049	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP050	GKNP-02	LP	E031S022CA	5	N3	0,00	neg
TP051	GKNP-02	LP	E031S022CA	10	N2/3	0,00	neg
TP052	GKNP-02	LP	E031S022CA	10	N2/3	0,00	neg
TP053	GKNP-02	LP	E031S022CA	10	N2/3	0,00	neg
TP054	GKNP-02	LP	E031S022CA	10	N2/3	0,00	neg

TP055	GKNP-02	LP	E031S022CA	10	N2/3	0,00	neg		
TP056	GKNP-02	LP	E031S022CA	10	N2/3	0,00	neg		
TP057	GKNP-02	LP	E031S022CA	10	N2/3	0,00	neg		
TP058	GKNP-02	LP	E031S022CA	10	N2/3	0,00	neg		
TP059	GKNP-02	LP	E031S022CA	15	N1/2	0,00	neg		
TP060	GKNP-02	LP	E031S022CA	15	N1/2	45,98	neg	NT	
TP061	GKNP-02	LP	E031S022CA	15	N1/2	0,00	neg		
TP062	GKNP-02	LP	E031S022CA	15	N1/2	0,00	neg		
TP063	GKNP-16	MP	E031S025BC	1	Ad	0,00	neg		
TP064	GKNP-16	MP	E031S025BC	1	Ad	0,00	neg		
TP065	GKNP-16	MP	E031S025BC	1	Ad	0,00	neg		
TP066	GKNP-16	MP	E031S025BC	1	Ad	0,00	neg		
TP067	GKNP-16	MP	E031S025BC	1	Ad	0,00	neg		
TP068	GKNP-16	MP	E031S025BC	1	Ad	0,00	neg		
TP069	GKNP-15	MP	E031S025BA	5	N3/4	0,00	neg	POS	OK137122
TP070	GKNP-15	MP	E031S025BA	5	N3/4	0,00	neg		neg
TP071	GKNP-15	MP	E031S025BA	5	N3/4	0,00	neg	POS	OK137123
TP072	GKNP-15	MP	E031S025BA	5	N3/4	0,00	neg		
TP073	GKNP-15	MP	E031S025BA	5	N3/4	0,00	neg		

TP074	GKNP-15	MP	E031S025BA	5	N3/4	0,00	neg			
TP075	GKNP-15	MP	E031S025BA	5	N3/4	0,00	neg			
TP076	GKNP-15	MP	E031S025BA	5	N3/4	0,00	neg			
TP077	GKNP-15	MP	E031S025BA	5	N3/4	0,00	neg			
TP078	GKNP-15	MP	E031S025BA	5	N3/4	0,00	neg			
TP079	GKNP-14	MP	E031S025BA	1	Ad	0,00	neg			
TP080	GKNP-14	MP	E031S025BA	2	N4/Ad	0,00	neg			
TP081	GKNP-14	MP	E031S025BA	2	Ad	0,00	neg	POS	OK137124	
TP082	GKNP-14	MP	E031S025BA	2	Ad	0,00	neg			neg
TP083	GKNP-14	MP	E031S025BA	4	N3	0,00	neg	POS	OK137125	
TP084	GKNP-14	MP	E031S025BA	1	Ad	0,00	neg			
TP085	GKNP-14	MP	E031S025BA	1	Ad	0,00	neg			
TP086	GKNP-14	MP	E031S025BA	1	Ad	0,00	neg			
TP087	GKNP-14	MP	E031S025BA	2	Ad	0,00	neg			
TP088	GKNP-14	MP	E031S025BA	2	Ad	0,00	neg			
TP089	GKNP-14	MP	E031S025BA	2	Ad	0,00	neg			
TP090	GKNP-14	MP	E031S025BA	2	Ad	0,00	neg			
TP091	GKNP-14	MP	E031S025BA	2	Ad	0,00	neg			
TP092	GKNP-14	MP	E031S025BA	10	N2	0,00	neg			

TP093	GKNP-14	MP	E031S025BA	100	N1	0,00	neg		
TP094	GKNP-14	MP	E031S025BA	100	N1	0,00	neg		
TP095	GKNP-14	MP	E031S025BA	100	N1	0,00	neg		
TP096	GKNP-14	MP	E031S025BA	100	N1	0,00	neg		
TP097	GKNP-14	MP	E031S025BA	100	N1	0,00	neg		
TP098	GKNP-14	MP	E031S025BA	1	Ad	0,00	neg		
TP099	GKNP-14	MP	E031S025BA	2	Ad	0,00	neg		
TP100	GKNP-14	MP	E031S025BA	2	Ad	0,00	neg		
TP101	GKNP-14	MP	E031S025BA	2	Ad	0,00	neg		
TP102	GKNP-14	MP	E031S025BA	2	Ad	0,00	neg		
TP103	GKNP-14	MP	E031S025BA	15	N2/3	0,00	neg		
TP104	GKNP-17	MP	E031S025BD	2	Ad	0,00	neg		
TP105	GKNP-17	MP	E031S025BD	2	Ad	0,00	neg		
TP106	GKNP-17	MP	E031S025BD	2	Ad	0,00	neg		
TP107	GKNP-17	MP	E031S025BD	2	Ad	0,00	neg		
TP108	GKNP-17	MP	E031S025BD	2	Ad	0,00	neg	POS	NS
TP109	GKNP-17	MP	E031S025BD	2	Ad	0,00	neg		neg
TP110	GKNP-17	MP	E031S025BD	15	N3/4	0,00	neg	POS	OK136972
TP111	GKNP-17	MP	E031S025BD	10	N2	0,00	neg		

TP112	GKNP-17	MP	E031S025BD	10	N2	0,00	neg		
TP113	GKNP-17	MP	E031S025BD	10	N2	0,00	neg	POS	OK136974
TP114	GKNP-17	MP	E031S025BD	15	N1	0,00	neg	POS	OK136975
TP115	GKNP-17	MP	E031S025BD	15	N1	0,00	neg		
TP116	GKNP-17	MP	E031S025BD	15	N1	0,00	neg		
TP117	GKNP-17	MP	E031S025BD	15	N1	0,00	neg		
TP118	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP119	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP120	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP121	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP122	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP123	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP124	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP125	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP126	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP127	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP128	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP129	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		
TP130	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg		

TP131	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg			
TP132	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg			
TP133	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg			
TP134	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg			
TP135	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg			
TP136	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg			
TP137	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg			
TP138	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg			
TP139	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg			
TP140	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg			
TP141	GKNP-16	MP	E031S025BC	5	N4/Ad	0,00	neg	POS	OK136976	
TP142	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg			neg
TP143	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	POS		
TP144	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg			
TP145	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg			
TP146	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg			
TP147	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg			
TP148	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg			
TP149	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg			

TP150	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP151	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP152	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP153	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP154	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP155	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP156	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP157	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP158	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP159	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP160	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP161	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP162	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP163	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP164	GKNP-16	MP	E031S025BC	2	Ad	41,59	neg	NT
TP165	GKNP-16	MP	E031S025BC	2	Ad	0,00	neg	
TP166	GKNP-16	MP	E031S025BC	15	N1/2	0,00	neg	
TP167	GKNP-16	MP	E031S025BC	15	N1/2	0,00	neg	
TP168	GKNP-16	MP	E031S025BC	15	N1/2	0,00	neg	

TP169	NWP-05	NWP	E026S025CA	10	N2	0,00	neg							
TP170	NWP-05	NWP	E026S025CA	10	N2	0,00	neg							
TP171	NWP-05	NWP	E026S025CA	10	N2	0,00	neg							
TP172	NWP-05	NWP	E026S025CA	10	N2	0,00	neg							
TP173	NWP-05	NWP	E026S025CA	10	N2	0,00	neg							
TP174	NWP-05	NWP	E026S025CA	5	N2/3	0,00	neg							
TP175	NWP-05	NWP	E026S025CA	5	N2/3	0,00	neg							
TP176	NWP-05	NWP	E026S025CA	5	N2/3	26,37	POS	POS	OK148638	POS	OM327321	POS	OK136980	
TP177	NWP-05	NWP	E026S025CA	5	N2/3	0,00	neg							
TP178	NWP-05	NWP	E026S025CA	5	N2/3	0,00	neg							
TP179	NWP-05	NWP	E026S025CA	5	N2/3	0,00	neg							
TP180	NWP-05	NWP	E026S025CA	10	N2	0,00	neg							
TP181	NWP-05	NWP	E026S025CA	10	N2	36,11	POS	POS	OK148639	POS	OM327322	POS	OK136981	
TP182	NWP-05	NWP	E026S025CA	10	N2	0,00	neg							
TP183	NWP-05	NWP	E026S025CA	5	N2/3	0,00	neg							
TP184	NWP-05	NWP	E026S025CA	5	N2/3	26,03	POS	POS	OK148640	POS	OM327323	POS	OK136982	
TP185	NWP-05	NWP	E026S025CA	5	N2/3	0,00	neg							
TP186	NWP-05	NWP	E026S025CA	5	N2/3	0,00	neg							
TP187	NWP-05	NWP	E026S025CA	5	N2/3	22,44	POS	POS	OK148641	POS	OM327324		neg	

TP189	NWP-05	NWP	E026S025CA	5	N2/3	0,00	neg								
TP190	NWP-05	NWP	E026S025CA	5	N2/3	0,00	neg								
TP191	NWP-05	NWP	E026S025CA	5	N2/3	0,00	neg								
TP192	NWP-05	NWP	E026S025CA	2	N4/Ad	0,00	neg								
TP193	NWP-05	NWP	E026S025CA	2	N4/Ad	23,15	POS	POS	OK148642	POS	OM327325	POS	OK136984		
TP194	NWP-05	NWP	E026S025CA	2	N4/Ad	0,00	neg								
TP195	NWP-05	NWP	E026S025CA	2	N4/Ad	0,00	neg								
TP196	NWP-05	NWP	E026S025CA	2	N4/Ad	0,00	neg								
TP197	NWP-05	NWP	E026S025CA	2	N4/Ad	0,00	neg								
TP198	NWP-05	NWP	E026S025CA	2	N4/Ad	0,00	neg								
TP199	NWP-05	NWP	E026S025CA	2	N4/Ad	0,00	neg								
TP200	NWP-05	NWP	E026S025CA	1	Ad	0,00	neg								
TP201	NWP-05	NWP	E026S025CA	1	Ad	0,00	neg								
TP202	NWP-05	NWP	E026S025CA	1	Ad	0,00	neg								
TP203	NWP-05	NWP	E026S025CA	1	Ad	25,99	POS	POS	OK148643	POS	OM327326	POS	OK136985		
TP204	NWP-05	NWP	E026S025CA	15	N1/2	42,03	neg	NT							
TP205	NWP-05	NWP	E026S025CA	15	N1/2	0,00	neg								
TP206	NWP-05	NWP	E026S025CA	15	N1/2	0,00	neg								
TP207	GP-02	GP	E028S025AD	2	N4/Ad	0,00	neg					POS	OK136986		
TP208	GP-02	GP	E028S025AD	2	N4/Ad	0,00	neg								neg

TP209	GP-02	GP	E028S025AD	10	N2/3	0,00	neg						POS	OK136987	
TP210	GP-02	GP	E028S025AD	15	N1/2	0,00	neg								
TP211	GP-02	GP	E028S025AD	15	N1	0,00	neg								
TP212	GP-02	GP	E028S025AD	1	Ad	0,00	neg								
TP213	NWP-04	NWP	E026S025CA	1	Ad	0,00	neg								
TP214	NWP-04	NWP	E026S025CA	5	N3/4	27,65	POS	POS	OK148644		OM327327	POS	OK136988		
TP215	NWP-03	NWP	E026S025AA	1	Ad	29,36	POS	POS	OK148645	POS	OM327328	POS	OK136989		
TP216	NWP-03	NWP	E026S025AA	100	N1	0,00	neg								
TP217	NWP-03	NWP	E026S025AA	1	Ad	0,00	neg								
TP218	NWP-03	NWP	E026S025AA	1	Ad	0,00	neg								
TP219	NWP-03	NWP	E026S025AA	1	Ad	0,00	neg								
TP220	NWP-03	NWP	E026S025AA	1	Ad	0,00	neg								
TP221	NWP-03	NWP	E026S025AA	2	N4/Ad	26,21	POS	POS	OK148646	POS	OM327329	POS	OK136990	neg	
TP222	NWP-03	NWP	E026S025AA	5	N4/Ad	40,23	neg	NT				POS	OK136991		
TP223	NWP-03	NWP	E026S025AA	8	N4/Ad	0,00	neg								
TP224	NWP-03	NWP	E026S025AA	8	N4/Ad	41,20	neg	NT							
TP225	ECP-01	ECP	E024S033AB	1	Ad	0,00	neg								neg
TP226	ECP-01	ECP	E024S033AB	1	Ad	0,00	neg					POS	OK136992		
TP227	LP-03	LP	E026S024DB	1	Ad	0,00	neg								
TP228	LP-03	LP	E026S024DB	1	Ad	0,00	neg								
TP229	LP-03	LP	E026S024DB	1	Ad	0,00	neg								
TP230	LP-03	LP	E026S024DB	1	Ad	26,87	POS	Weak POS	OK148647			Weak	OK136993		
TP231	LP-03	LP	E026S024DB	1	Ad	0,00	neg								
TP232	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg								

TP233	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP234	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP235	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP236	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP237	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP238	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP239	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP240	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP241	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP242	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP243	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP244	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP245	LP-03	LP	E026S024DB	2	N4/Ad	36,44	neg	neg		neg
TP246	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP247	LP-03	LP	E026S024DB	2	N4/Ad	0,00	neg			
TP248	LP-03	LP	E026S024DB	18	N2	0,00	neg			
TP249	LP-03	LP	E026S024DB	5	N3/4	0,00	neg			
TP250	LP-03	LP	E026S024DB	5	N3/4	0,00	neg			
TP251	LP-03	LP	E026S024DB	5	N3/4	26,34	Repeat =neg	neg	POS	OK136995
TP252	LP-03	LP	E026S024DB	5	N3/4	0,00	neg			
TP253	LP-03	LP	E026S024DB	5	N3/4	0,00	neg			
TP254	AENP-03	ECP	E025S033DB	10	UR	0,00	neg		POS	MZ411417
TP255	AENP-03	ECP	E025S033DB	10	UR	0,00	neg		POS	OK136996
TP256	AENP-03	ECP	E025S033DB	20	UR	0,00	neg		POS	OK136997
TP257	AENP-03	ECP	E025S033DB	20	UR	0,00	neg		POS	OK136998
TP258	AENP-03	ECP	E025S033DB	20	UR	0,00	neg		POS	OK136999
TP259	AENP-01	ECP	E025S033BD	4	UR	0,00	neg		POS	MZ411418

TP260	AENP-02	ECP	E025S033BD	5	UR	0,00	neg		POS	MZ411419
TP261	AENP-02	ECP	E025S033BD	5	UR	0,00	neg		POS	OK137001
TP262	AENP-02	ECP	E025S033BD	5	UR	0,00	neg		POS	OK137002
TP263	AENP-02	ECP	E025S033BD	5	UR	0,00	neg		POS	OK137003
TP264	AENP-02	ECP	E025S033BD	5	UR	0,00	neg		POS	OK137004
TP265	AENP-02	ECP	E025S033BD	5	UR	0,00	neg		POS	OK137005
TP266	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137006
TP267	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137007
TP268	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137008
TP269	AENP-02	ECP	E025S033BD	10	UR	0,00	neg	neg	POS	OK137009
TP270	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137010
TP271	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137011
TP272	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137012
TP273	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137013
TP274	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137014
TP275	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137015
TP276	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137016
TP277	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137017
TP278	AENP-02	ECP	E025S033BD	10	UR	0,00	neg		POS	OK137018

TP279	AENP-02	ECP	E025S033BD	10	UR	0,00	neg				POS	OK137019	
TP280	AENP-02	ECP	E025S033BD	10	UR	0,00	neg				POS	OK137020	
TP281	AENP-02	ECP	E025S033BD	20	UR	0,00	neg				POS	OK137021	
TP282	AENP-02	ECP	E025S033BD	12	UR	0,00	neg				POS	OK137022	
TP283	GKNP-18	MP	E031S025BD	1	Ad	0,00	neg						
TP284	GKNP-18	MP	E031S025BD	2	N4/Ad	0,00	neg						
TP285	GKNP-18	MP	E031S025BD	2	N4/Ad	0,00	neg						
TP286	GKNP-18	MP	E031S025BD	100	N1	0,00	neg						
TP287	GKNP-18	MP	E031S025BD	2	Ad	0,00	neg						
TP288	GKNP-18	MP	E031S025BD	2	Ad	25,52	POS	POS	OK148648		POS	OK137023	
TP289	GKNP-18	MP	E031S025BD	1	Ad	28,11	POS	Repeat= neg			Weak	OK137024	neg
TP290	GKNP-18	MP	E031S025BD	13	N2/3	27,94	POS	POS	OK148649		POS	OK137025	
TP291	GKNP-18	MP	E031S025BD	1	Ad	0,00	neg						
TP292	GKNP-18	MP	E031S025BD	100	N1	0,00	neg						
TP293	GKNP-18	MP	E031S025BD	2	N2/3	21,94	POS	Repeat= POS	OK148680		Weak	OK137026	
TP294	GKNP-18	MP	E031S025BD	5	N3	0,00	neg						
TP295	GKNP-18	MP	E031S025BD	10	N1/2	0,00	neg						
TP296	GKNP-18	MP	E031S025BD	13	N1/2	28,08	POS	POS	OK148650				
TP297	GKNP-06	LP	E031S023CD	11	N2	0,00	neg						

TP298	GKNP-06	LP	E031S023CD	3	N4/Ad	0,00	neg			
TP299	GKNP-06	LP	E031S023CD	1	Ad	0,00	neg			
TP300	GKNP-06	LP	E031S023CD	1	Ad	0,00	neg			
TP301	GKNP-06	LP	E031S023CD	1	Ad	0,00	neg			
TP302	GKNP-06	LP	E031S023CD	1	Ad	0,00	neg			
TP303	GKNP-06	LP	E031S023CD	1	Ad	0,00	neg			
TP304	GKNP-06	LP	E031S023CD	1	Ad	0,00	neg			
TP305	GKNP-06	LP	E031S023CD	1	Ad	0,00	neg			
TP306	GKNP-06	LP	E031S023CD	15	N1	0,00	neg	POS	OK137027	
TP307	GKNP-06	LP	E031S023CD	20	N1	0,00	neg			neg
TP308	GKNP-05	LP	E031S023DC	3	N3	0,00	neg			
TP309	GKNP-05	LP	E031S023DC	21	N1/2	0,00	neg			
TP310	GKNP-05	LP	E031S023DC	10	N2	0,00	neg			
TP311	GKNP-05	LP	E031S023DC	10	N2	0,00	neg			
TP312	GKNP-05	LP	E031S023DC	10	N2	0,00	neg			
TP313	GKNP-05	LP	E031S023DC	10	N2	0,00	neg			
TP314	GKNP-05	LP	E031S023DC	10	N2	0,00	neg			
TP315	GKNP-05	LP	E031S023DC	10	N2	0,00	neg	POS	OK137028	
TP316	GKNP-05	LP	E031S023DC	10	N2/3	0,00	neg			neg

TP317	GKNP-05	LP	E031S023DC	5	UR	0,00	neg		
TP318	GKNP-05	LP	E031S023DC	2	N4/Ad	0,00	neg	POS	OK137029
TP319	GKNP-05	LP	E031S023DC	2	N4/Ad	0,00	neg		
TP320	GKNP-05	LP	E031S023DC	2	N4/Ad	0,00	neg		
TP321	GKNP-05	LP	E031S023DC	2	N4/Ad	0,00	neg		
TP322	GKNP-05	LP	E031S023DC	2	N4/Ad	0,00	neg		
TP323	GKNP-04	LP	E031S023CC	1	Ad	0,00	neg		
TP324	GKNP-04	LP	E031S023CC	1	Ad	0,00	neg		
TP325	GKNP-04	LP	E031S023CC	1	Ad	0,00	neg		
TP326	GKNP-04	LP	E031S023CC	1	Ad	0,00	neg		
TP327	GKNP-04	LP	E031S023CC	1	Ad	0,00	neg		
TP328	GKNP-04	LP	E031S023CC	1	Ad	0,00	neg		
TP329	GKNP-04	LP	E031S023CC	1	Ad	0,00	neg		
TP330	GKNP-04	LP	E031S023CC	1	Ad	0,00	neg		
TP331	GKNP-04	LP	E031S023CC	1	Ad	0,00	neg		
TP332	GKNP-04	LP	E031S023CC	1	Ad	0,00	neg		
TP333	GKNP-04	LP	E031S023CC	1	Ad	0,00	neg	POS	OK137030
TP334	GKNP-04	LP	E031S023CC	10	N2	0,00	neg		neg
TP335	GKNP-04	LP	E031S023CC	10	N2	0,00	neg		

TP336	GKNP-04	LP	E031S023CC	10	N2	0,00	neg
TP337	GKNP-04	LP	E031S023CC	10	N2	0,00	neg
TP338	GKNP-04	LP	E031S023CC	5	N2/3	0,00	neg
TP339	GKNP-04	LP	E031S023CC	5	N2/3	0,00	neg
TP340	GKNP-04	LP	E031S023CC	5	N2/3	0,00	neg
TP341	GKNP-04	LP	E031S023CC	5	N2/3	0,00	neg
TP342	GKNP-04	LP	E031S023CC	5	N2/3	0,00	neg
TP343	GKNP-04	LP	E031S023CC	5	N2/3	0,00	neg
TP344	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg
TP345	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg
TP346	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg
TP347	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg
TP348	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg
TP349	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg
TP350	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg
TP351	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg
TP352	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg
TP353	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg
TP354	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg

TP355	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP356	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP357	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP358	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP359	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP360	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP361	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP362	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP363	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP364	GKNP-04	LP	E031S023CC	2	N4/Ad	36,43	Repeat =neg	neg	POS OK137031
TP365	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP366	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP367	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP368	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP369	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP370	GKNP-04	LP	E031S023CC	2	N4/Ad	0,00	neg		
TP371	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg		
TP372	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg		
TP373	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg		

TP374	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP375	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP376	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP377	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP378	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP379	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP380	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP381	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP382	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP383	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP384	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP385	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP386	GKNP-04	LP	E031S023CC	3	N3/4	0,00	neg												
TP387	NCP-04	NCP	E024S028CB	5	N3	0,00	neg										POS	OK137032	
TP388	NCP-04	NCP	E024S028CB	18	N1/2	0,00	neg										POS	OK137033	
TP389	NCP-04	NCP	E024S028CB	4	UR	25,04	POS	POS	OK148651								POS	OK137034	neg
TP390	NCP-04	NCP	E024S028CB	22	UR	0,00	neg										POS	OK137035	
TP391	NCP-05	NCP	E023S028DD	21	N1/2	0,00	neg										POS	OK137036	
TP392	NCP-05	NCP	E023S028DD	5	N2/3	0,00	neg												neg
TP393	NCP-03	NCP	E024S028BC	2	N4/Ad	0,00	neg												
TP394	NCP-03	NCP	E024S028BC	18	N1/2	0,00	neg												
TP395	NCP-03	NCP	E024S028BC	3	N3	0,00	neg												
TP396	NCP-03	NCP	E024S028BC	10	N1	0,00	neg												
TP397	NCP-03	NCP	E024S028BC	13	N1/2	0,00	neg												

TP398	NCP-03	NCP	E024S028BC	2	N4/Ad	0,00	neg									
TP399	NCP-03	NCP	E024S028BC	2	N4/Ad	0,00	neg					POS	OK137037			
TP400	NCP-03	NCP	E024S028BC	5	N2/3	0,00	neg								neg	
TP401	NCP-03	NCP	E024S028BC	2	N1/2	39,38	neg					POS	NS			
TP402	NWP-02	NWP	E026S025AA	7	N1/3	0,00	neg					POS	OK137038		neg	
TP403	NWP-01	NWP	E026S024CD	8	N1/2, Ad	0,00	neg									
TP404	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg									
TP405	NWP-01	NWP	E026S024CD	1	Ad	28,34	POS	POS	OK148652	POS	OM327307	POS	OK137039			
TP406	NWP-01	NWP	E026S024CD	1	Ad	41,79	neg	NT								
TP407	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg								neg	
TP408	NWP-01	NWP	E026S024CD	1	Ad	28,96	POS	POS	OK148653	POS	OM327308	POS	OK137040		neg	
TP409	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg									
TP410	NWP-01	NWP	E026S024CD	2	N4/Ad	0,00	neg									
TP411	NWP-01	NWP	E026S024CD	3	N3/4	0,00	neg									
TP412	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg									
TP413	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg									
TP414	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg									
TP415	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg									
TP416	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg									
TP417	NWP-01	NWP	E026S024CD	100	N1	0,00	neg									
TP418	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg									

TP419	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg							
TP420	NWP-01	NWP	E026S024CD	2	N4/Ad	0,00	neg							
TP421	NWP-01	NWP	E026S024CD	2	N4/Ad	0,00	neg							
TP422	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg							
TP423	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg							
TP424	NWP-01	NWP	E026S024CD	1	Ad	0,00	neg							
TP425	NWP-01	NWP	E026S024CD	1	Ad	28,27	POS	POS	OK148654	POS	OM327309	POS	OK137041	
TP426	NWP-01	NWP	E026S024CD	2	N4/Ad	0,00	neg							
TP427	NWP-01	NWP	E026S024CD	2	N4/Ad	0,00	neg							
TP428	FSP-03	FSP	E024S029BD	8	N1/N3	30,13	POS	POS	OK148655			POS	OK137042	
TP429	FSP-02	FSP	E025S029AC	10	N2/3	36,53	Repeat =neg	neg				POS	NS	
TP430	FSP-02	FSP	E025S029AC	10	N2/3	42,15	neg	NT						neg
TP431	FSP-02	FSP	E025S029AC	10	N2/3	27,80	POS	POS	OK148656	POS	OM327310	POS	OK137043	
TP432	FSP-02	FSP	E025S029AC	10	N2/3	41,74	neg	NT						
TP433	FSP-02	FSP	E025S029AC	10	N2/3	0,00	neg					POS	OK137044	
TP434	FSP-02	FSP	E025S029AC	20	N1	41,90	neg	NT						
TP435	FSP-02	FSP	E025S029AC	18	N1	0,00	neg							
TP436	NCP-02	NCP	E024S028AD	13	N1	27,99	POS	POS	OK148657	POS	OM327330	POS	OK137045	
TP437	NCP-02	NCP	E024S028AD	10	N2	45,87	neg	NT						
TP438	NCP-02	NCP	E024S028AD	10	N2	0,00	neg							
TP439	NCP-02	NCP	E024S028AD	10	N2	0,00	neg							
TP440	NCP-02	NCP	E024S028AD	10	N2	42,01	neg							
TP441	NCP-02	NCP	E024S028AD	10	N2	0,00	neg							
TP442	NCP-02	NCP	E024S028AD	3	N4/Ad	0,00	neg							
TP443	NCP-02	NCP	E024S028AD	3	N4/Ad	28,22	POS	POS	OK148658	POS	OM327331	POS	OK137046	

TP444	NCP-02	NCP	E024S028AD	3	N4/Ad	27,21	POS	POS	OK148659			POS	OK137047	neg
TP445	NCP-02	NCP	E024S028AD	3	N4/Ad	25,77	POS	Repeat= neg				POS	OK323968	
TP446	NCP-02	NCP	E024S028AD	3	N4/Ad	24,30	Repeat= neg							
TP447	NCP-01	NCP	E024S028BC	1	Ad	22,92	POS	POS	OK148660			POS	OK137048	
TP448	NCP-01	NCP	E024S028BC	1	Ad	0,00	neg							
TP449 ⁴	NCP-01	NCP	E024S028BC	3	N4/Ad	24,20	POS	POS	OK148661/ OK148662	POS	OM327332	POS	OK137049	
TP450	NCP-01	NCP	E024S028BC	3	N4/Ad	0,00	neg							
TP451	NCP-01	NCP	E024S028BC	3	N4/Ad	0,00	neg							
TP452	NCP-01	NCP	E024S028BC	3	N4/Ad	0,00	neg							
TP453	NCP-01	NCP	E024S028BC	3	N4/Ad	0,00	neg							
TP454	NCP-01	NCP	E024S028BC	3	N4/Ad	41,90	neg	neg						
TP455	NCP-01	NCP	E024S028BC	3	N4/Ad	0,00	neg							
TP456	NCP-01	NCP	E024S028BC	3	N4/Ad	0,00	neg							
TP457	NCP-01	NCP	E024S028BC	3	N4/Ad	40,89	neg	NT						
TP459 ⁴	NCP-01	NCP	E024S028BC	3	N4/Ad	29,23	POS	POS	OK148663/ OK148664	POS	OM327311	POS	OK137050	
TP460	NCP-01	NCP	E024S028BC	3	N4/Ad	0,00	neg							
TP461	NCP-01	NCP	E024S028BC	3	N4/Ad	0,00	neg							
TP462	NCP-01	NCP	E024S028BC	3	N4/Ad	37,21	POS	Repeat= neg				POS	OK137051	
TP463	NCP-01	NCP	E024S028BC	3	N4/Ad	44,17	neg	NT						
TP464	NCP-01	NCP	E024S028BC	3	N4/Ad	0,00	neg							
TP465	NCP-01	NCP	E024S028BC	3	N4/Ad	21,88	POS	POS	OK148665	POS	OM327317	POS	OK137052	
TP466	NCP-01	NCP	E024S028BC	3	N4/Ad	24,75	POS	POS	OK148666	POS	OM327312			
TP467	NCP-01	NCP	E024S028BC	3	N4/Ad	27,17	POS	Repeat= POS	OK148667	POS	OM327333	POS	OK137053	
TP468	NCP-01	NCP	E024S028BC	3	N4/Ad	30,96	POS	POS	OK148668			POS	OK137054	
TP469	NCP-01	NCP	E024S028BC	3	N4/Ad	24,58	POS	POS	OK148669	POS	OM327318	POS	OK137055	
TP470	NCP-01	NCP	E024S028BC	13	N1/2	0,00	neg							
TP471	NCP-01	NCP	E024S028BC	15	N1/2	0,00	neg							
TP472	NCP-01	NCP	E024S028BC	15	N1/2	42,29	neg	NT						

TP473	NCP-01	NCP	E024S028BC	15	N1/2	41,12	neg	NT						
TP474	NCP-01	NCP	E024S028BC	15	N1/2	26,20	POS	POS	OK148670				POS	OK137056
TP475	NCP-01	NCP	E024S028BC	10	N2	43,10	neg	NT						
TP476	NCP-01	NCP	E024S028BC	10	N2	25,66	POS	POS	OK148671				POS	OK137057
TP477	NCP-01	NCP	E024S028BC	10	N2	21,11	POS	POS	OK148672	POS	OM327334		POS	OK137058
TP478	NCP-01	NCP	E024S028BC	10	N2	20,23	POS	POS	OK148681				POS	OK137059
TP479	NCP-01	NCP	E024S028BC	10	N2	0,00	neg							
TP480 ⁴	NCP-01	NCP	E024S028BC	10	N2	21,32	POS	POS	OK148673/ OK148674				POS	OK137060
TP481	NCP-01	NCP	E024S028BC	10	N2	38,67	neg	NT						
TP482	NCP-01	NCP	E024S028BC	10	N2	39,73	neg	NT						
TP483	NCP-01	NCP	E024S028BC	10	N2	0,00	neg							
TP484	NCP-01	NCP	E024S028BC	5	N2/3	24,15	POS	POS	OK148675	POS	OM327335		POS	OK137061
TP485	NCP-01	NCP	E024S028BC	5	N2/3	0,00	neg							
TP486	NCP-01	NCP	E024S028BC	5	N2/3	22,27	POS	Repeat= POS	OK148682	POS	OM327313		POS	OK137062
TP487	NCP-01	NCP	E024S028BC	5	N2/3	0,00	neg							
TP488	NCP-01	NCP	E024S028BC	5	N2/3	25,36	POS	POS	OK148676	POS	OM327336		POS	OK137063
TP489	NCP-01	NCP	E024S028BC	5	N2/3	41,75	neg	NT						
TP490	NCP-01	NCP	E024S028BC	5	N2/3	32,51	POS	Weak POS	OK148677				POS	OK137064
TP491	NCP-01	NCP	E024S028BC	5	N2/3	0,00	neg							
TP492	NCP-01	NCP	E024S028BC	5	N2/3	23,73	POS	POS	OK148678	POS	OM327337		POS	OK137065
TP493	NCP-01	NCP	E024S028BC	5	N2/3	0,00	neg							
TP494	NCP-01	NCP	E024S028BC	5	N2/3	23,69	POS	POS	OK148679	POS	OM327338		POS	OK137066
TP495	NCP-01	NCP	E024S028BC	5	N2/3	0,00	neg							
TP496	NCP-01	NCP	E024S028BC	5	N2/3	0,00	neg							
TP497	NCP-01	NCP	E024S028BC	5	N2/3	0,00	neg							
TP498	NCP-01	NCP	E024S028BC	5	N2/3	0,00	neg							
TP499	GKNP-10	MP	E031S024BC	2	N4/Ad	0,00	neg							
TP500	GKNP-10	MP	E031S024BC	2	N4/Ad	0,00	neg							
TP501	GKNP-10	MP	E031S024BC	2	N4/Ad	0,00	neg							

TP502	GKNP-10	MP	E031S024BC	2	N4/Ad	0,00	neg
TP503	GKNP-10	MP	E031S024BC	5	N2/3	0,00	neg
TP504	GKNP-10	MP	E031S024BC	5	N2/4	0,00	neg
TP505	GKNP-10	MP	E031S024BC	1	N1/2	0,00	neg
TP506	GKNP-10	MP	E031S024BC	1	N1/2	0,00	neg
TP507	GKNP-13	MP	E031S024DB	1	Ad	0,00	neg
TP508	GKNP-13	MP	E031S024DB	2	N4/Ad	0,00	neg
TP509	GKNP-13	MP	E031S024DB	2	N4/Ad	0,00	neg
TP510	GKNP-13	MP	E031S024DB	2	N4/Ad	0,00	neg
TP511	GKNP-13	MP	E031S024DB	5	N2/3	0,00	neg
TP512	GKNP-13	MP	E031S024DB	5	N2/3	0,00	neg
TP513	GKNP-13	MP	E031S024DB	5	N2/3	0,00	neg
TP514	GKNP-13	MP	E031S024DB	10	N1/2	0,00	neg
TP515	GKNP-13	MP	E031S024DB	10	N1/2	0,00	neg
TP516	GKNP-13	MP	E031S024DB	10	N1/2	0,00	neg
TP517	GKNP-13	MP	E031S024DB	20	N1/2	0,00	neg
TP518	GKNP-13	MP	E031S024DB	20	N1/2	0,00	neg
TP519	GKNP-13	MP	E031S024DB	20	N1/2	0,00	neg
TP520	GKNP-13	MP	E031S024DB	20	N1/2	0,00	neg

TP521	GKNP-13	MP	E031S024DB	22	N1/2	0,00	neg
TP522	GKNP-09	MP	E031S024BC	1	Ad	0,00	neg
TP523	GKNP-09	MP	E031S024BC	1	Ad	0,00	neg
TP524	GKNP-09	MP	E031S024BC	2	N4/Ad	0,00	neg
TP525	GKNP-09	MP	E031S024BC	2	N4/Ad	0,00	neg
TP526	GKNP-09	MP	E031S024BC	2	N4/Ad	0,00	neg
TP527	GKNP-09	MP	E031S024BC	5	N3/4	0,00	neg
TP528	GKNP-09	MP	E031S024BC	5	N3/4	0,00	neg
TP529	GKNP-09	MP	E031S024BC	10	N1/2	0,00	neg
TP530	GKNP-09	MP	E031S024BC	10	N1/2	0,00	neg
TP531	GKNP-09	MP	E031S024BC	17	N1/2	0,00	neg
TP532	GKNP-08	MP	E031S024BA	1	Ad	0,00	neg
TP533	GKNP-08	MP	E031S024BA	2	N4/Ad	0,00	neg
TP534	GKNP-08	MP	E031S024BA	2	N4/Ad	0,00	neg
TP535	GKNP-08	MP	E031S024BA	2	N4/Ad	0,00	neg
TP536	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP537	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP538	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP539	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg

TP540	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP541	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP542	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP543	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP544	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP545	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP546	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP547	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP548	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP549	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP550	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP551	GKNP-08	MP	E031S024BA	5	N3/4	0,00	neg
TP552	GKNP-08	MP	E031S024BA	10	N1/2	0,00	neg
TP553	GKNP-08	MP	E031S024BA	10	N1/2	0,00	neg
TP554	GKNP-08	MP	E031S024BA	10	N1/2	0,00	neg
TP555	GKNP-08	MP	E031S024BA	10	N1/2	0,00	neg
TP556	GKNP-08	MP	E031S024BA	10	N1/2	0,00	neg
TP557	GKNP-08	MP	E031S024BA	10	N1/2	0,00	neg
TP558	GKNP-12	MP	E031S024DD	1	Ad	0,00	neg

TP559	GKNP-12	MP	E031S024DD	5	N3/4	0,00	neg
TP560	GKNP-12	MP	E031S024DD	5	N3/4	0,00	neg
TP561	GKNP-12	MP	E031S024DD	10	N2/3	0,00	neg
TP562	GKNP-12	MP	E031S024DD	10	N2/3	0,00	neg
TP563	GKNP-12	MP	E031S024DD	10	N2/3	0,00	neg
TP564	GKNP-12	MP	E031S024DD	20	N1/2	0,00	neg
TP565	GKNP-12	MP	E031S024DD	20	N1/2	0,00	neg
TP566	GKNP-11	MP	E031S024DB	1	Ad	0,00	neg
TP567	GKNP-11	MP	E031S024DB	1	Ad	0,00	neg
TP568	GKNP-11	MP	E031S024DB	2	N4/Ad	0,00	neg
TP569	GKNP-11	MP	E031S024DB	2	N4/Ad	0,00	neg
TP570	GKNP-11	MP	E031S024DB	2	N4/Ad	0,00	neg
TP571	GKNP-11	MP	E031S024DB	2	N4/Ad	0,00	neg
TP572	GKNP-11	MP	E031S024DB	2	N4/Ad	0,00	neg
TP573	GKNP-11	MP	E031S024DB	2	N4/Ad	0,00	neg
TP574	GKNP-11	MP	E031S024DB	2	N4/Ad	0,00	neg
TP575	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg
TP576	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg
TP577	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg

TP578	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP579	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP580	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP581	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP582	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP583	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP584	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP585	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP586	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP587	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP588	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP589	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP590	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP591	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP592	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP593	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	
TP594	GKNP-11	MP	E031S024DB	3	N4/Ad	0,00	neg	neg
TP595	GKNP-11	MP	E031S024DB	5	N3/4	0,00	neg	neg
TP596	GKNP-11	MP	E031S024DB	5	N3/4	0,00	neg	neg

TP597	GKNP-11	MP	E031S024DB	5	N3/4	0,00	neg							neg
TP598	GKNP-11	MP	E031S024DB	5	N3/4	0,00	neg							neg
TP599	GKNP-11	MP	E031S024DB	10	N3/4	0,00	neg							neg
TP600	GKNP-11	MP	E031S024DB	10	N3/4	0,00	neg							neg
TP601	GKNP-11	MP	E031S024DB	10	N3/4	0,00	neg							neg
TP602	GKNP-07	MP	E031S024BA	2	N4/Ad	25,50	POS	POS	OK148683			POS	OK137074	neg
TP603	GKNP-07	MP	E031S024BA	2	N4/Ad	0,00	neg					POS	OK137075	neg
TP604	GKNP-07	MP	E031S024BA	2	N4/Ad	0,00	neg					POS	OK137076	neg
TP605	GKNP-07	MP	E031S024BA	2	N4/Ad	0,00	neg					POS	OK137077	
TP606	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg					POS	OK137078	
TP607	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg					POS	OK137079	
TP608	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg					POS	OK137080	
TP609	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg					POS	OK137081	
TP610	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg					POS	OK137082	
TP611	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg					POS	OK137083	
TP612	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg					POS	OK137084	
TP613	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg					POS	OK137085	
TP614	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg					POS	OK137086	
TP615	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg					POS	OK137087	

TP616	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg				POS	OK137088	
TP617	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg				POS	OK137089	
TP618	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg				POS	OK137090	
TP619	GKNP-07	MP	E031S024BA	3	N4/Ad	42,44	neg				POS	OK137091	
TP620	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg				POS	OK137092	
TP621	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg				POS	OK137093	
TP622	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg				POS	OK137094	
TP623	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg				POS	OK137095	
TP624	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg				POS	OK137096	
TP625	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg				POS	OK137097	
TP626	GKNP-07	MP	E031S024BA	3	N4/Ad	0,00	neg				POS	OK137098	
TP627	GKNP-07	MP	E031S024BA	5	N2/3	24,77	POS	POS	OK148684		POS	OK137099	neg
TP628	GKNP-07	MP	E031S024BA	5	N2/3	0,00	neg				POS	OK137100	
TP629	GKNP-07	MP	E031S024BA	5	N2/3	0,00	neg				POS	OK137101	
TP630	GKNP-07	MP	E031S024BA	5	N2/3	0,00	neg				POS	OK137102	
TP631	GKNP-07	MP	E031S024BA	5	N2/3	0,00	neg				POS	OK137103	
TP632	GKNP-07	MP	E031S024BA	5	N2/3	0,00	neg				POS	OK137104	
TP633	LP-01	LP	E029S022CB	1	N4/Ad	0,00	neg				POS	OK137105	neg
TP634	LP-01	LP	E029S022CB	1	N4/Ad	0,00	neg				POS	OK137106	neg
TP635	LP-01	LP	E029S022CB	1	N4/Ad	0,00	neg						neg
TP636	LP-01	LP	E029S022CB	1	N4/Ad	0,00	neg				POS	OK137107	neg

TP637	LP-01	LP	E029S022CB	1	N4/Ad	0,00	neg				POS	OK137108	neg
TP638	LP-01	LP	E029S022CB	1	N4/Ad	0,00	neg				POS	OK137109	neg
TP639	LP-01	LP	E029S022CB	1	N4/Ad	0,00	neg				POS	OK137110	neg
TP641	GP-01	GP	E028S025BC	1	N4/Ad	0,00	neg				POS	OK137111	neg
TP642	GP-01	GP	E028S025BC	1	N4/Ad	0,00	neg				POS	OK137112	neg
TP643	GP-01	GP	E028S025BC	1	N4/Ad	0,00	neg				POS	OK137113	neg
TP644	GP-01	GP	E028S025BC	1	N4/Ad	0,00	neg				POS	OK137114	neg
TP645	GP-01	GP	E028S025BC	4	N3	0,00	neg						neg
TP646	GP-04	GP	E028S025BA	1	N4/Ad	0,00	neg				POS	OK137115	neg
TP647	GP-04	GP	E028S025BA	2	N4/Ad	0,00	neg				POS	OK137116	neg
TP648	GP-04	GP	E028S025BA	2	N4/Ad	0,00	neg				POS	OK137117	neg
TP649	GP-04	GP	E028S025BA	2	N4/Ad	39,53	neg				POS	OK137118	neg
TP650	GP-04	GP	E028S025BA	3	N3	0,00	neg				POS	OK137119	neg
TP651	GP-05	GP	E028S025BC	1	N4/Ad	0,00	neg				POS	OK137120	neg
TP652	GP-05	GP	E028S025BC	1	N4/Ad	0,00	neg				POS	OK137121	neg
TP653	GP-05	GP	E028S025BC	1	N4/Ad	0,00	neg				POS	NS	neg
TP654	GP-05	GP	E028S025BC	7	N1/N1	0,00	neg				POS	NS	neg
				3									
TP-RICHT	LP-02	LP	E027S024CA	2	N3	0,00	neg						
TP-RDPLT	GP-03	GP	E028S025CB	1	N3	0,00	neg				POS	OK137126	
TP001MNP	MNP-01	NCP	E024S029AB	5	N2/3	0,00	neg				POS	OK137127	
TP002MNP	MNP-01	NCP	E024S029AB	5	N2/3	0,00	neg				POS	OK137128	
TP003MNP	MNP-01	NCP	E024S029AB	1	N4	0,00	neg				POS	OK137129	
TP004MNP	MNP-01	NCP	E024S029AB	2	N1/2	0,00	neg				POS	OK137130	
TP005MNP	MNP-01	NCP	E024S029AB	10	N1/2	23,54	POS	POS	NS		POS	OK137131	neg
TP006MNP	MNP-01	NCP	E024S029AB	5	N2/3	0,00	neg				POS	OK137132	
TP007MNP	MNP-01	NCP	E024S029AB	10	N1/2	39,78	neg				POS	OK137133	

TP008M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg					POS	OK137134	
TP009M NP	MNP- 01	NCP	E024S029AB	5	N2/3	0,00	neg					POS	OK137135	
TP010M NP	MNP- 01	NCP	E024S029AB	1	Ad	0,00	neg					POS	OK137136	
TP011M NP	MNP- 01	NCP	E024S029AB	10	N1/2	25,03	POS	POS	OK148685	POS	OM327319	POS	OK137137	neg
TP012M NP	MNP- 01	NCP	E024S029AB	2	N1/2	0,00	neg					POS	NS	
TP013M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg					POS	OK137138	
TP014M NP	MNP- 01	NCP	E024S029AB	5	N2/3	0,00	neg					POS	OK137139	
TP015M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg					POS	OK137140	
TP016M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg					POS	OK137141	
TP017M NP	MNP- 01	NCP	E024S029AB	5	N2/3	34,18	POS	neg	NS			POS	OK137142	neg
TP018M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg					POS	OK137143	
TP019M NP	MNP- 01	NCP	E024S029AB	3	N2/3	0,00	neg					POS	OK137144	
TP020M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	39,12	neg	neg				POS	OK137145	
TP021M NP	MNP- 01	NCP	E024S029AB	7	N2/3	0,00	neg					POS	OK137146	
TP022M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg					POS	OK137147	
TP023M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg					POS	OK137148	
TP024M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg					POS	OK137149	
TP025M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg					POS	OK137150	
TP026M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg					POS	OK137151	

TP027M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg						POS	OK137152	
TP028M NP	MNP- 01	NCP	E024S029AB	UR	N1	0,00	neg						POS	OK137153	
TP029M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg						POS	OK137154	
TP030M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg						POS	OK137155	
TP031M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg						POS	OK137156	
TP032M NP	MNP- 01	NCP	E024S029AB	1	Ad	0,00	neg						POS	OK137157	
TP033M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg						POS	OK137158	
TP034M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg						POS	OK137159	
TP035M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg						POS	OK137160	
TP036M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	41,71	neg						POS	OK137161	
TP037M NP	MNP- 01	NCP	E024S029AB	5	N2/3	25,94	POS	POS	OK148686	POS	OM327320	POS	OK137162	Weak	
TP038M NP	MNP- 01	NCP	E024S029AB	10	N1/2	23,61	POS	POS	OK148688	POS	OM327314	POS	OK137163	neg	
TP039M NP	MNP- 01	NCP	E024S029AB	UR	N1	0,00	neg						POS	OK137164	
TP040M NP	MNP- 01	NCP	E024S029AB	10	N1/2	26,63	POS	POS	OK148687	POS	OM327315	POS	OK137165	neg	
TP041M NP	MNP- 01	NCP	E024S029AB	UR	N1	0,00	neg						POS	OK137166	
TP042M NP	MNP- 01	NCP	E024S029AB	UR	N1	0,00	neg						POS	OK137167	
TP043M NP	MNP- 01	NCP	E024S029AB	UR	N1	0,00	neg						POS	OK137168	
TP044M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg						POS	OK137169	
TP045M NP	MNP- 01	NCP	E024S029AB	UR	N1	0,00	neg						POS	OK137170	

TP046M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	42,14	neg					POS	OK137171
TP047M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg					POS	OK137172
TP048M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg					POS	OK137173
TP049M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg					POS	OK137174
TP050M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg					POS	OK137175
TP051BN F	FSP-01	FSP	E024S029BD	2	N3	0,00	neg					POS	OK137069
TP052M NP	MNP- 01	NCP	E024S029AB	2	N4/Ad	0,00	neg					POS	OK137176
TP053M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg					POS	OK137177
TP054M NP	MNP- 01	NCP	E024S029AB	3	N3	0,00	neg					POS	OK137178
TP055M NP	MNP- 01	NCP	E024S029AB	10	N1/2	0,00	neg					POS	OK137179
DKT1	NCP-06	NCP	E022S028CC	10	UR	0,00	neg						
DKT2	NCP-06	NCP	E022S028CC	10	UR	0,00	neg						
DKT3	NCP-06	NCP	E022S028CC	11	UR	29,01	POS	POS	OL870945	POS	OM327316	POS	OL870945

¹ ECP = Eastern Cape Province; FSP = Free State Province; GP = Gauteng Province; LP = Limpopo Province; MP = Mpumalanga Province; NCP = Northern Cape Province; NWP = North West Province.

² QDGC = Quarter degree grid cell.

³ N = Nymph; Ad = Adult.

⁴ Three tick pools yielded two ASFV genotypes.

NT = Not tested.

NS = No sequence available.

UR = unrecorded.