SUSCEPTIBILITY OF SUS SCROFA TO RIFT VALLEY FEVER VIRUS: IMPLICATIONS FOR ANIMAL AND HUMAN HEALTH IN AFRICA

By

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DECLARATION

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

SIGNATURE: 

DATE: ...27 October 2022…….

DEDICATION

This work is dedicated to my late mother, Dorah Mohlala-Lubisi, for the human being, mother, guardian, mentor and friend she was, and the guardian angel she will forever be.

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LIST OF ABBREVIATIONS

ABC	Avidin-Biotinylated peroxidase Complex
ACI	August Copenhagen Irish
AEC	Animal Ethics Committee
AF	Aborted Foetus
AGID	Agar Gel Immuno-Diffusion
AP	Apparent Prevalence
API	Animal Production Institute
APP	Acute Phase Proteins
APR	Acute-Phase Response
APTT	Activated Partial Thromboplastin Time
ARC	Agricultural Research Council
BHK	Baby Hamster Kidney
BLAST	Basic Local Alignment Search Tool
BSL	Biological Safety Level
BT	Bluetongue
CAE	Cost-Effectiveness Analysis
CPE	Cytopathic Effect
CRP	C-Reactive Protein
CT	Threshold Cycle
CV	Coefficient of Variation
DA	Dark Agouti
IFN	Interferon
I/N	Intranasal
I/P	Intra-peritoneal
I/T	Intratesticular
I/D	International
IU	International Units
I/V	Intra -venous
KNP	Kruger National Park
KZN	Kwa-Zulu Natal
L	Lamb
L segment	Large segment

LD50	Lethal Dose 50
LFT	Lateral FlowTest
LP	Limpopo Province
LS	Lactating Sow
M	M segment
MIC	Mouse intracranial
MIP	Mouse intraperitoneal
MIPLD50	Mouse intraperitoneal Lethal Dose 50
MP	Mpumalanga Province
mRNA	messenger RNA
N	Nucleocapsid
NA	Not Analysed
NC	Northern Cape
ND	Not Done
NS	Non-structural
NEAA	Non-Essential Amino Acids
NGO	Non-Governmental Organisations
NP	Nucleoprotein
N/S	Not Stated
NT	Not Tested
NW	North West
OBP	Onderstepoort Biological Products
OD	Optical Density
OIE	Office International des Epizooties
OVR	Onderstepoort Veterinary Research
P	Piglet (newborn)
PBS	Phospahate Buffered Saline
P/C	Per Cutaneous
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
PPE	Personal Protective Equipment
PRNT	Plaque Reduction Neutralisation Test
PS	Pregnant Sow

PT	Prothrombin time
PTE	Proximal Tubular Epithelial
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
ROC	Receiver Operating Characteristic
RT-PCR	Reverse-Transcription Polymerase Chain Reaction
RVF	Rift Valley Fever
RVFV	Rift Valley Fever Virus
S	Small segment
SAA	Serum Amyloid A
SB	Stillborn
S/C	Subcutaneous
SD	Standard Deviation
SP	Sucking Piglet
SST	Sea Surface Temperatures
TADP	Transboundary Animal Diseases Programme
TC	Tissue Culture
TMB	Tetramethylbenzidine
UTR	Untranslated regions
VI	Virus Isolation
VNT	Virus Neutralisation Test
W	Weaner
WAD	West African Dwarf
WC	Western Cape
WF	Wistar-Furth
WHO	World Health Organisation
ZH	Zagazig Hospital
WF	Wistar-Furth
WHO	World Health Organisation
ZH	Zagazig Hospital

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SUMMARY

Rift Valley fever (RVF) is a vector-borne viral zoonotic disease which affects domestic ruminants and camels. It is characterised by abortion storms and neonatal deaths in animals, and has severe economic impacts. While humans mostly present with self-limiting flu-like symptoms, the disease can progress to more severe signs and even death. Following the discovery of RVF in the early 1930s, initial investigations considered various animal species, including pigs, for susceptibility to the disease by infecting them with the causative agent, RVF virus (RVFV). Using various routes of infection, transmission, pathogenesis, and discernible immune response data established that sheep, goats, cattle and mice were susceptible to infection, but that pigs were not. Also generated were field epidemiological data involving different invertebrate vectors, and various mosquito and animal species. The information gathered was used to devise the current control measures, which mainly focus on ruminants to the general exclusion of domestic pigs. However, reports on the susceptibility of domestic pigs (*Sus scrofa domesticus*) to RVFV were conflicting and those which concluded that pigs were amenable to infection did not describe the clinicopathological course of infection in this species. Given the epidemiological links of pigs to known RVF hosts in mixed farming set-ups in endemic countries, this study aimed to use current diagnostic laboratory techniques such as blocking enzyme linked immuno-sorbent assays (ELISA), virus neutralisation test (VNT), histopathology and immunohistochemistry, conventional and real time RT-PCR, and sequencing, as well as infection experiments and electron microscopy to determine: (i) the effect of RVFV infection on the pregnant sow; (ii) the effect of RVFV infection on neonatal piglets and weaners and (iii) to establish and apply methods that are suitable for assesing RVF seroprevalence in domestic and wild suids in order to establish their natural exposure to the virus. Results showed that pregnant sows can abort following infection with high doses of RVFV under experimental conditions, and that neonatal pigs and weaners can also be successfully infected, although the infection is sub-clinical. Evaluation of the widely used blocking ELISA for determining anti-RVFV antibodies was shown to be less sensitive than the VNT in experimentally infected pigs. Finally, application of VNT revealed that domestic pigs and warthogs in South Africa were naturally exposed to RVFV and seroconverted to the virus, albeit at lower rates than ruminants. These findings demonstrate the merit of using pigs in RVF biomedical research and contribute to our understanding of the role of suids in the epidemiology of RVF in Africa. The results further revealed the VNT to be the best serological test to use for sero-surveillance of RVF in suids, in the absence of faster and more efficient assays. Demonstration of RVFV in aborted and newborn piglets highlights the need for exercising safety precautions when handling not only ruminant, but also swine aborted materials during outbreaks, to avoid possible virus transmisson to humans. Further studies to isolate and quantify RVFV from blood and oronasal and faecal secretions of infected pigs will assist with determining their potential to infect vector mosquitoes and shed virus in

the environment, since viral RNA was shown to persist for at least 28 days post infection in such secretions in this study.

CHAPTER I

LITERATURE REVIEW

1.1 History of Rift Valley fever

In July 1930, Daubney et al. (1931) of the Division of Veterinary Research, Kenya Colony, received a request to assist with the diagnosis of a disease that caused abortion storms and new born lamb mortalities on a farm in the Rift Valley. The farm in question was an open bush area of approximately 30 000 acres on which Merino sheep were raised successfully. Sheep were normally synchronised to lamb during the months of October and November, but in this particular year, the manager of the farm decided to have the lambing season during July and August. The decision was made for purposes of capitalizing on the unusual rainfalls prevailing at the time, which ensured availability of good grazing throughout the dry season. Traditionally, Kenya has four rainy seasons; the first characterised by low average rainfalls occurs between January and February. The second, the “long rains” season is characterised by the highest rainfalls and occurs from March to May, while a second in rains from October to December is the “short rains”. The intervening low rainfall period of July to September is the dry season (Ayugi et al., 2016). The sheep on the farm had not previously suffered from severe livestock diseases, and the only diseases which were recorded on the farm were: i) sheep pox, which was controlled by vaccination; ii) heartwater, which was almost eradicated at the time due to regular dipping of cattle on the premises; and iii) bluetongue (BT), which was seasonal and appeared towards the end of the short rains and normally disappeared by the end of January. However, due to the abnormally high rainfalls experienced in 1930, BT persisted even in the month of July (Daubney et al., 1931).

The abortions which occurred at the end of June were initially attributed to BT, since BT clinical signs were produced at the laboratory on inoculation of susceptible sheep with blood from recently aborted ewes. Nonetheless, not all blood from ewes which aborted produced BT on inoculation of susceptible sheep, raising the suspicion that something else may be responsible for the observed clinical signs and mortalities (Daubney et al., 1931). Non-pregnant or non-aborting ewes were also observed to either die suddenly without having shown any prior clinical signs or to appear ill for a few hours and die shortly thereafter. Clinically ill animals presented with thick muco-purulent nasal discharges, occasional bloody stools, anorexia, lethargy and disinclination to rise, and normal temperatures (Daubney et al., 1931). By mid-August 1930, the lamb and ewe mortality rates were 3500 and 1200 respectively. A link between the hyperacute lamb and ewe disease, where the latter seemed to recover following abortion, was made. Illness and mortalities continued until

December, while they stopped among flocks which were moved to higher altitudes of about 70008500 feet (2134 – 2591 metres).

In order to prevent or curb future losses, it was crucial that the cause of the abortions and neonatal mortalities be identified. Bacteriological studies were performed since the liver and mesenteric lesions observed in the dead animals were highly suggestive of a *Salmonella*-type infection, but results proved that the cause was unlikely to be bacterial in nature (Daubney et al., 1931). Transmission studies using the bloods of lambs and ewes that succumbed to the disease as inoculum managed to reproduce the disease in recipient lambs and ewes respectively, proving the infectious nature of the disease. Cross immunity studies using recovered animals and injecting them with infectious material from other animals *e.g* recovered lamb inoculated with material from sick ewe and *vice versa* resulted in all animals being protected from clinical disease and proved the relatedness of the ewe and lamb disease.The agent was proven to be present in blood, plasma, liver and spleen of infected animals, and filterable. Infective doses of 0.04 to 0.0002cc/ml were found to induce disease.

Natural transmission was investigated through observation of diseased and in-contact susceptible animals in the same enclosures, and placing three groups of sheep (n = 19) in the field in pens with different insect protection: group 1 sheep (n = 6) were in cages covered by mosquitoe nets at all times; group 2 (n = 5) were allowed to graze without protection during the day, but were covered at night; and group 3 (n = 8) also grazed in the open during the day and were not completely covered at night. It was observed that sheep which were artificially inoculated with virus and reacting were housed with susceptible ones, but the latter did not contract the disease. In addition, artificially infected lactating ewes and lambs failed to transmit the virus to their offspring and dams respectively. Lambs fostered by ewes that had lost their newborns to the disease also never became infected. In the field, the animals which were completely protected from insects and those which were protected from insect bites at night did not develop any signs of disease and were amenable to experimental infection at the end of the study, except for one in group 2, which developed a reaction and was later shown to be resistant to infection. One of the sheep in the group 3 exposed to insect bites at all times died of enzootic hepatitis, and the majority, except for two, developed fever and were resistant to infection at the end. The results lead researchers to conclude that the virus was transmitted by an insect which could not pass through a mosquito net (Daubney et al., 1931). Mosquitoes were the logical vectors of the causative agent of the disease since they were abundant at the lower levels of the valley where the outbreak had started, and mortalities and abortions were significantly reduced when the animals were moved to higher altitude.

Mosquitoes were caught and homogenized, inoculated into susceptible sheep, and any reactions followed up with sub-inoculations of other susceptible animals and immunity tests. Among the mosquitoes investigated for habouring the virus were *Culex pipiens* and *C. fatigans*, *Anopheles squamosus* and *A. mauritianus*, and *Taeniorhynchus brevipalpis.* It was concluded that the most probable vector was *T. brevipalpis* from the progressive leucocyte changes observed in one laboratory-born lamb (Daubney et al., 1931).

1.2 Susceptible animals

Even though clinical signs of the disease which was subsequently called Rift Valley fever (RVF) were first seen in pregnant ewes and neonatal lambs aged between 1 and 7 days, it was imperative that other animals be investigated for susceptibility to the causative agent, RVF virus (RVFV), in the interest of understanding the epidemiology of the disease, and devising control measures. Earlier workers successfully inoculated various species of animals using the subcutaneous (S/C), intradermal (I/D), intraperitoneal (I/P), intracerebral (I/C), intratesticular (I/T), per cutaneous (P/C), intranasal (I/N), intravenous (I/V) and per conjunctival routes. Infection could not be established orally or through direct contact in earlier experiments (Daubney et al., 1931; Findlay, 1932a; Findlay & Daubney, 1931; Easterday et al., 1962; Scott, 1963). The minimum infective dose in mice was determined by making 1/100 to 1/100,000,000 dilutions of infected blood in phosphate buffer at a pH of 7.2. It was found that doses of 1/1,000,000 were lethal to mice, and that dilutions of 1/100 and 1/1000 also killed epilated and scarified mice (Findlay, 1932a). Table 1.1 (b – e) highlights findings on the susceptibility and clinical studies done on some domestic and wild animals, and on humans.

Table 1.1a A summary of the susceptibility of various vertebrate species to RVFV infection, as determined by Findlay and Daubney (1931) when using infected human and lamb blood as inoculum

No	Species	Scientific name	Susceptibility
1	Human	*Homo sapiens*	++
2	Monkey	*Macaca mulata*	++
3	Cat	*Felis catus domesticus*	+
4	Cow	*Bos primigenius*	++
5	Sheep	*Ovis aries*	+++
6	Lamb	*Ovis aries*	++++
7	Goat	*Capra aegagrus hircus*	++
8	Horse	*Equus caballus*	-
9	Pig	*Sus scrofa domesticus*	-
10	Mouse	*Mus*	++++
11	Wood mouse	*Apodemus sylvaticus*	++++
12	Field Vole	*Microtus agrestis*	++++
13	Dormouse	*Gliridae*	++++
14	Golden hamster	*Mesocricetus auratus*	++++
15	Rat	*Rattus rattus*	+++
16	Grey Squirrel	*Sciurus carolinensis*	++
17	Rabbit	*Oryctolagus cuniculus*	+/-
18	Guinea pig	*Cavia porcellus*	-
19	Mongoose	*Herpestidae*	-
20	Hedgehog	*Erinaceinae*	-
21	Tortoise	*Testudinidae*	-
22	Frog	*Anura*	-
23	Hen	*Gallus gallus domesticus*	-
24	Pigeon	*Columbidae*	-
25	Canary	*Serinus canaria domestica*	-
26	Parakeet	*Melopsittacus undulatus*	-
14	Golden hamster	*Mesocricetus auratus*	++++
15	Rat	*Rattus rattus*	+++
16	Grey Squirrel	*Sciurus carolinensis*	++

++++: nearly always fatal; +++: approximately 50% mortality rate; ++: severe but non-fatal reaction; +: slight reaction; +/-: virus may survive for a while in the blood, but, without causing any reaction; -: not susceptible

Table 1.1b Summary of experiments conducted to determine susceptibility of main domestic animals and humans to RVFV according to Daubney et al. (1931) using blood as inoculum. No information regarding seroconversion was provided

No	Animal and number	Inoculum	Dose	Route	Rise in temp.	Typical clinical signs	Virus isolated / Antigen present
1	*Sheep (*Ovis aries*)_ (n=several)	Blood	0.040.0002 cc	I/V	Yes	Yes	Yes
2	Goat (*Capra aegagrus hircus*) (n=1)	N/S	N/S	N/S	N/S	Yes	N/S
3	Cow (*Bos Taurus*) (n=6)	Blood	N/S	N/S	Yes	N/S	N/S
4	Man (*Homo sapiens*) (n=1)	Blood	N/S	N/S	Yes	No	N/S
5	^Horse (*Equus caballus*) (n=1)	N/S	N/S	N/S	N/S	No	N/S
6	^Pig (*Sus scrofa domesticus*) (n=2)	N/S	N/S	N/S	N/S	No	N/S

I/V: intravenous; N/S: Not stated; Temp: Temperature; *: Characteristic histopathological signs observed; ^: Uncharacteristic histopathological signs observed

Table 1.1c Rift valley fever virus pathogenicity studies conducted by Findlay (1932a) using blood from a variety of species as inoculum

No	Animal and number	Dose	Route	Rise in temp.	Clinical signs	Sero-conversion	Virus isolated/ Antigen present
1	Mice (*Mus musculus*) (N>700)	0.2c.cm	I/P	Normal to decrease	Yes (Death)	N/S	Yes (Blood infective)
2	Rats (*Rattus rattus*) (n=43)	0.05 - 3c.cm	I/P; S/C; I/C and I/T	Normal to decrease	Yes (Death)	N/S	Yes (Blood infective)
3	Monkey (*Macaca mulatta*) (n=14)	0.2c.cm – 0.5c.cm	I/P; I/C; S/C and I/N	Yes	No	N/S	Yes (Blood and liver infective)
4	Rabbits (*Oryctolagu s cuniculus*) (n=12)	N/S	I/P; I/V; S/C; I/D; and I/C	No	No	N/S	No
5	Guinea pigs (*Cavia porcellus*) (n=25)	N/S	I/P; S/C; I/D; and I/C	No	No	N/S	No
6	Canaries (*Serinus canaria domestica)*	N/S	N/S	No	No	N/S	No

No	Animal and number	Dose	Route	Rise in temp.	Clinical signs	Sero conversion	Virus isolated/ Antigen present
7	Goat (*Capra aegagrus hircus*) (n=2)	2 and 2.5 c.cm	I.P	Yes	Yes	N/S	Yes (Blood infective)
8	Sheep – ewe (*Ovis aries*)_ (n=1)	5c.cm	I.P	Yes	No	N/S	Yes (Blood infective)
9	*Sheeplamb (Ovis aries)_ (n=4)	0.2c.cm	I.P	Yes	Yes (Death)	Yes (DPI10)	Yes (Blood infective)
10	Man (*Homo sapiens*) (n=3)	N/S	I/N	Yes	Yes	Yes (DPI 9)	Yes (Blood infective)
11	Cat (*Felis catus*) (n=3)	0.25c.cm	I.P	Yes	Yes	N/S	Yes (Blood infective)
12	Wood mice (*Apodemus sylvaticus*) (n=10)	0.2c.cm	I.P	N/S	Yes (Death)	N/S	Yes (Blood infective)
13	Field vole (*Microtus agrestis*) (n=6)	0.2c.cm	I.P	N/S	Yes (Death)	N/S	Yes (Blood infective)
14	Dormice (*Gliridae*) (n=3)	0.2c.cm	I.P	N/S	Yes (Death)	N/S	Yes (Blood infective)
15	Golden hamster (*Mesocricet us auratus*) (n=2)	0.5c.cm	I.P	N/S	Yes (Death)	N/S	Yes (Blood infective)
16	Grey squirrel (*Sciurus carolinensis*) (n=2)	0.3c.cm	I.P	No	Yes (Death)	N/S	Yes (Blood infective)
17	Mongoose (*Herpestida e*)	N/S	N/S	No	No	N/S	No
18	Hedgehog (*Erinaceina e*)	N/S	N/S	No	No	N/S	No
19	Reptiles (*Reptilia*)	N/S	N/S	No	No	N/S	No
20	Amphibians (*Amphibia*)	N/S	N/S	No	No	N/S	No

No	Animal and number	Dose	Route	Rise in temp.	Clinical signs	Seroconversion	Virus isolated/ Antigen present
21	Birds (*Aves*)	N/S	N/S	No	No	N/S	No

N/S: Not stated; I/P: Intraperitoneal; I/C: Intracerebral; S/C: Subcutaneous; I/N: Intranasal; I/D: Intradermal; I/V: Intravenous; DPI: Days post infection; Temp: Temperature; *: Characteristic histopathological signs observed

Table 1.1d Clinical and viraemia studies performed on lambs, calves and pigs (Easterday et al., (1962) using the serum of a sheep infected with the Van Wyk strain of RVFV

Exp. No.	Animal and number	Dose	Route	Rise in temp.	Clinical signs	Seroconversion	Virus isolated / Antigen Present
1	Calf (*Bos Taurus*) (n=2)	200 MIP LD_{50}	I.P	Yes	Yes	N/S	Yes
2	Calf (*Bos Taurus*) (n=2)	100 MIP LD_{50}	I.D	Yes	Yes	N/S	Yes
3	Calf (*Bos Taurus*) (n=2)	$10^{-7.3}$ MP MP LD_{50}	I.D	Yes	Yes	N/S	Yes
4	Calf (*Bos Taurus*) (n=2)	$10^{-4.3}$ MIP LD_{50}	I.D	Yes	Yes	N/S	Yes
5	Calf (*Bos Taurus*) (n=3)	1=12 MIPLD_{50}, 2 and 3 = 1.2 MIPLD_{50}	I.D	Yes	Yes	N/S	Yes
6	Calf (*Bos Taurus*) (n=3)	1 and 2 = 0.1MIPLD_{50}; 3=0.01MIPLD_{50}	I.D	Yes (1 and 2)	Yes (1 and 2)	N/S	Yes (1and 2)
				No (3)	No (3)	N/S	No (3)
7	[a]Goat (*Capra aegagrus hircus*) (n=3)	150 MIPLD_{50}	I.P	Yes	No	N/S	~Yes
8	*[b]Goat (*Capra aegagrus hircus*) (n=6)	1 MIPLD_{50} (n=2)	I.P	Yes	N/S	N/S	Yes
		10 MIPLD_{50} (n=2)		Yes	N/S	N/S	Yes
		100 MIPLD_{50} (n=2)		Yes	N/S	N/S	Yes
9	^Pig (*Sus scrofa domesticus*) (n=3)	150 MIPLD_{50} (n=2)	I.P	No	No	No	No
		1500 MIPLD_{50} (n=1)		No	No	N/S	No

Exp: Experiment; MIP: Mouse intraperitoneal; LD: Lethal dose; I.P: Intra-peritoneal; ID: Intradermal; N/S: Not stated; Temp: Temperature; *: Characteristic histopathological signs observed; ^: Uncharacteristic histopathological signs observed; ~: No virus detected in milk. Calves were male and female Holstein Friesians (<1 week old), Goats, Toggenburg and Saanen breeds, all males except 1 lactating female (a: Doe was 2-4 years old; kids 4 months old; b. Kids 12 hours to 6 days old), and Pigs, Hormel types, males and females, (no age specified)

Table 1.1e Susceptibility of domestic pigs to RVFV using the serum of a sheep infected with the Van Wyk (Scott, 1963) and ZH 501 (Clarke et al., 2021) strains of RVFV as the inoculum

No.	Animal and number	Dose	Route	Rise in temp.	Typical clinical signs	Seroconversion	Viraemia/ Virus isolated/ Antigen present
1	Pig (*Sus scrofa domesticus*) (n=3)	10^{-2} to 10^{-3} hamster LD_{50} (Van Wyk strains)	I/P	N/S	No	No	No
2	Pig (*Sus scrofa domesticus*) (n=3)	10^{-5} to 10^{-6} hamster LD_{50} (Van Wyk strains)	I/P	N/S	No	Yes (n = 3)	Yes (n = 1)
3	Pig (*Sus scrofa domesticus*) (n = 6)	10^{-5} pfu/ml (ZH 501 strain)	S/C	No	No	Yes (n = 6)	Yes (n = 4)

LD: Lethal dose; N/S: Not stated; I/P: Intra-peritoneal; Temp: Temperature. Pigs were of an undisclosed sex, large white breed, immature, and weighed 34-45kg (Scott, 1963); and landrace cross, males and females, approximately one month old (Clarke et al., 2021)

From the Table 1.1 summaries (1.1b to 1.1e) it is clear that only eleven pigs were infected with RVFV between 1931 and 1963, with only limited information being provided about the age and physiological status of the pigs. Moreover, no pregnant sows were included in the studies. Results indicated that pigs do not appear to be susceptible to infection as none displayed typical clinical signs of infection, with only three seroconverting and viral antigen demonstrated from only one of the eleven pigs infected.

1.3 Pathogenesis, Clinical Signs and Pathology

1.3.1 Mouse (*Mus musculus*)

Similar to observations made by earlier researchers, the liver was the major target organ of RVFV in mice challenged subcutaneously (s/c) with 1000 plaque-forming units (PFU) of RVFV strain Zagazig Hospital 501 (ZH501) in a total volume of 100 µL. At necropsy, hepatitis was exhibited, including congestion and haemorrhage in the spleen which was of normal size and mottled, lymph nodes, especially those of the mesentry, large intestines, kidneys and brain, but not in the jejunum. Inflammation of the transverse colon was occasionally observed. Mesenteric vessels were congested, with haemorrhagic foci seen on the mesentery and omentum (McGavran & Easterday, 1963; Smith et al., 2010).

Microscopically, lesions characterised by fulminant hepatitis with coagulative necrosis, leaving the portal space unaffected, congestion and haemorrhages were exhibited (McGavran & Easterday, 1963; Smith et al., 2010). Viral antigens started accumulating in hepatocytes on days post infection (DPI) 2 and increased by DPI 3, inducing apoptosis in hepatocytes (Smith et al., 2010). Swelling of the endothelial cells was seen, but not of the Kupffer cells. Presence of eosinophilic intranuclear inclusion bodies in infected hepatocytes was a common feature (Findlay & Daubney, 1931). Apoptosis of lymphocytes was found in the thymus, spleen, lymph nodes and mucosa-associated lymphoid tissues. In some of the mice that survived acute viral hepatitis, a sharp decrease in viral antigens occurred at DPI 8 and no virus could be detected in the serum, liver, lung, pancreas, large intestine and ovaries. In the late stage of infection, however, lethal meningoencephalitis characterised by neuronal necrosis, microhaemorrhages, and perivascular cuffs occurred in mice that survived the acute hepatitis (Smith et al., 2010).

1.3.2 Rat (*Rattus*)

Clinically, different strains of rats displayed varying susceptibility to RVFV infection, even though the clinicopathological outcome of infection in the strains was seemingly dependent on the route of inoculation. It was shown that 10 to 15 week old inbred rats from the U.S. breeders exhibited three different responses to subcutaneous inoculation with the ZH501 strain of RVFV, where Wistar-Furth (WF) and Brown Norway strains showed high susceptibility to the virus and died within DPI 4 following infection with 5 pfu of virus, while the Fisher 344, Buffalo, Dark Agouti (DA) and Lewis strains were largely resistant to infection at titres of 5×10^5 pfu. Maax and August Copenhagen Irish (ACI) strains proved to be moderately susceptible at titres of 5×10^3 pfu to 5×10^5 pfu, and ascending paralysis was a common feature in these strains (Findlay & Howard, 1952; Peters & Slone, 1982). Recently, adult black rats (*Rattus rattus*, Tilbury strain) which were subcutaneously inoculated with 0.25 ml of 1 x 10^5 $TCID_{50}$/ml of RVFV 35/74 strain were shown to be clinically normal following infection (Stoek et al., 2022).

Viral tropism in the ACI and Maax strains was also different, in that the viruses were undetectable in the liver and blood, but 5 to 6 log pfu/g of viruses could be detected from brain tissue, irrespective of the presence of neutralising antibody in the serum. However, intracranial injection of RVFV consistently caused encephalitis in the ACI and Maax rats, including the resistant Lewis strain (Peters & Slone, 1982). In the study conducted by Stoek et al. (2022), the rats seroconverted. The blood, brain, lung, heart, liver, spleen, kidney, and intestines, and conjunctival, oral and rectal swabs were positive for RVFV RNA and live virus could be detected in all sample types except brain, heart and intestines.

At necropsy, hepatitis was the main lesion and cause of death in highly susceptible strains. Similar to mice, among other organs affected was the spleen, which was frequently of normal size and mottled in appearance. The mesenteric vessels were congested, and haemorrhagic foci were seen on the mesentery, omentum and mesenteric lymph nodes. Haemorrhages could be seen in the cardiac areas of the stomach, lungs, pericardium (left ventricle), testicles, uterus, and bladder. Stoek et al. (2022) only observed variable degrees of lung collapse with multifocal to coalescing white nodules, as typical RVF macroscopic lesions were absent.

Histopathological lesions in strains with ascending paralysis were mainly in the brain and spinal cord and were characterised as mild to severe necrotising encephalitis and encephalomyelitis with focal necrosis, neutrophilic infiltrate and perivascular cuffing primarily with lymphocytes (Peters & Slone, 1982). Stoek et al. (2022) observed mild to severe, multifocal, perivascular, subacute, lymphohistiocytic interstitial pneumonia associated with alveolar oedema and multifocal, intra-alveolar aggregations of hyperplastic, foamy alveolar macrophages and cholesterol clefts. Also seen was a single case of mild, focal, subacute, lymphohistiocytic and necrotising hepatitis with a few neutrophils. The perivascular pneumonia with alveolar oedema and hyperplasia of foamy alveolar macrophages found in the majority of infected, contact and negative control animals, was nonetheless suspected to have been caused by a co-existing pulmonary pneumocystosis due to its manifestation (Stoek et al., 2022).

1.3.3 Other rodents

a). Gerbil (*Gerbillinae*)

The Mongolian jird/gerbil (*Meriones unguiculatus*) which was investigated as a model for the encephalitic form of RVF, appeared to be resistant to RVFV infection with age, strain and dose of inoculum seemingly playing important roles in the degree of susceptibility and survival rates, as observed by Anderson et al. (1988), who subcutaneously inoculated different breeds and age groups with doses of 10^7, 10^5, 10^3, 10^1 pfu of ZH501strain of RVFV. Clinically, the Mongolian gerbil mostly presented with hind-limb paralysis, generalised weakness and wasting. There was minimal involvement of the liver following infection with RVFV in this species.

Histopathologically, minimal necrosis of hepatocytes was observed DPI 1 or 2 following subcutaneous inoculation. Focal necrotising encephalitis with neuronal necrosis, neutrophilic infiltration and perivascular cuffing were seen in the brain at a later stage (Anderson et al., 1988). Clinically normal gerbils were proven to develop mild, necrotising encephalitis, in the absence of detectable infectious virus.

b). Hamster (*Cricetinae*)

Death from severe liver necrosis following intraperitoneal inoculation with graded doses of strain ZH501 occurred after 2 to 3 days (Niklasson et al., 1984). Necropsy changes such as those seen in mice, *e.g* red-purple mottling of the liver, congestion of the mesenteric and omental vessels, and petechial haemorrhages of the mesenteric and omental glands, developed. Administration of low titres of neutralising antibodies protected hamsters from fatal liver necrosis, but, the animals succumbed to encephalitis and died by DPI 11 (Niklasson et al., 1984).

1.3.4 Man (*Homo sapiens*)

Human infections with RVFV are generally subclinical or mild, but acute clinical disease can develop. The incubation period in man is 5 to 6 days (Findlay, 1932a). The following clinical signs were observed: initially, shivering, undulating or biphasic fever of 39 degrees Celsius (normal is 36.5 - 37.5 degrees Celsius), malaise, anorexia, throbbing headaches, large-joint arthralgia (*e.g* knees, elbows, and shoulders) and a rise in pulse of up to 120 beats per minute (normal is 60 to 100 beats per minute). Involvement of the digestive system is manifested by nausea and vomition, and midepigastric pain and discomfort normally ensued. Constipation was common.

In a few patients, haematemesis and epistaxis occured, indicating development of the haemorrhagic form of the disease. Hepatomegaly, scleral icterus, secondary hyperhidrosis and delirium characterised the peak of the disease course (Madani et al., 2003; Strausbaugh et al., 1978).

Ocular and central nervous system involvement in the form of retinal vasculitis, photophobia, altered vision, and encephalitis were observed in the late stages of RVF infection (Kahlon et al., 2010). Leucocytosis affecting the polymorphonuclear leucocytes, followed by leukopenia usually persisted until convalescence in surviving patients. A small percentage of patients can develop a more severe form of disease in the form meningoencephalitis, hepatitis or renal failure, coupled with, or without retinitis, leading to death (Kasye et al., 2016).

1.3.5 Non-human primates

Three species of African monkey *i.e*. green guenon (*Cercopithecus callitrichus*), sooty mangabey (*Cercocebus fuliginosus*) and Patas guenon (*Erythrocebus patas*), did not exhibit any febrile reaction following inoculation with RVFV, even though viraemia was detected in the blood (Findlay, 1932b). Davies et al. (1972) reported that RVFV-infected baboons (*Papio anubis*) developed and maintained viraemia for 3 to 4 days following inoculation, without developing significant clinical signs. Four species of South American monkeys on the contrary developed a fever for 1 to 2 days following infection. These were two brown capuchin monkeys (*Cebus fatuellus* and *Cebus chrysopus*) and two common marmosets (*Callithrix jacchus* and *Callithrix penicillata*) (Findlay, 1932b).

Experiments performed in the early 1930s have shown that Rhesus macaques are moderately susceptible to RVFV. Following intraperitoneal or intranasal inoculations, the temperatures of virus infected macaques increased to 39–40°C from DPI 1 to 4, lasting 24 to 120 hours, while other animals did not display temperature increases (Findlay & Daubney, 1931). In experiments conducted by Peters et al. (1988) where 15 rhesus macaque monkeys were infected with RVFV strain ZH501 intravenously, only 3 showed overt signs of illness, while others remained clinically normal and only developed brief viraemia which peaked on DPI 2.

In a study performed by Morrill et al. (1990), 17 rhesus macaques were infected with RVFV ZH501 strain and three developed signs consistent with haemorrhagic fever and died, seven were clinically, but not fatally ill, and the remaining seven did not display any clinical signs. Increased interferon (IFN)-α levels in the serum of surviving and fatally infected animals were detected from 6 to 24 hours post infection (HPI) and 24 to 30 HPI respectively, and viraemia was detected prior to detection of the IFN-α in two of the three lethally-infected monkeys (Morrill et al., 1990).

The three sick macaques reported by Peters et al. (1988) were lethargic, weak and anorexic. Petechiae, ecchymoses and bleeding from nares, gums or venipuncture sites were observed. Activated partial thromboplastin time (APTT) and prothrombin time (PT) were prolonged and partially prolonged respectively, suggestive of deficiencies of coagulation factors and platelets. Findlay and Daubney (1931) reported that RVFV-infected macaques developed an undulating or biphasic fever of 38.5 to 41 degrees Celsius. Among the 14 macaques that survived infection with the RVFV ZH501 strain (Morrill et al. 1990), none developed signs of encephalitis or retinal complications after two months to two years.

In macaques, overt pathological signs were seen in the digestive system. The liver was light yellow in colour, with reddish mottling. Congestion of the mesenteric vessels and glands was noted, with scattered haemorrhagic spots in the mesentery. The glands had small haemorrhages in the cortex on sectioning (Findlay, 1932b).

Laboratory analysis of the samples collected by Findlay (1932b) revealed abnormalities associated with the immune system. Similar to the observations in man, leucocytosis affecting the polymorphonuclear leucocytes developed, followed by leukopenia which usually persisted until convalescence was seen. Microscopically, moderate focal or midzonal coagulative necrosis of the liver involving approximately a third of the hepatocytes was observed in the dead macaque monkeys. In the heart, necrosis in the myocardium of the ventricles was observed. In the kidneys, fibrin thrombi in the glomeruli and small intertubular vessels of the renal medulla were a constant feature. Mild

depletion of lymphocytes from the white pulp and deposition of eosinophilic amorphous fibrin-like material in the red pulp of the spleen were observed (Peters et al., 1988).

1.3.6 Sheep

Daubney et al. (1931) reported that RVF seemed to be more severe in neonatal lambs than adults, and generally, the older the lamb became, the less severe or pronounced the clinical signs were. In infant lambs of up to 1 week of age, high mortalities (95% - 100%) were observed. Lambs older than 1 week displayed lethargy, disinclination to move, anorexia, diarrhoea, nasal discharge and fever, increased prothrombin time, and some may die (Easterday et al., 1962). In adult animals, RVF is more pronounced in pregnant ewes, where abortion storms result. A small percentage of ewes would die from the disease. Breed seems to also play a role in the pathogenesis of RVF in sheep as demonstrated by Olaleye et al. (1996b) where they found some breeds to be more susceptible than others.

a). Gross pathology

i). Liver and Gall bladder

Foetuses: Livers were usually swollen and friable, and yellow or dark red, but necrotic foci or haemorrhage were not a common feature. The gall bladder did not show any gross lesions (Odendaal et al., 2020b).

Lambs: The liver was friable and appeared swollen and light yellow or yellowish-brown due to diffuse necrosis, or dark red due to severe congestion. The necrotic foci were prominent or masked by congestion in some cases. Oedema and occasional haemorrhages were present in the gallbladder of some lambs (Odendaal et al., 2020a).

Adults: Mottled red appearance due to presence of many ill-defined necrotic foci, and haemorrhage and oedema, were common in the liver and gallbladder respectively (Odendaal et al., 2019).

ii). Spleen

Foetuses, lambs and adults: No lesions were seen in the foetuses but occassional capsular and subcapsular haemorrhages were present in lambs and adults (Odendaal et al., 2019, 2020a, 2020b).

iii). Kidney

Foetuses, lambs and adults: No lesions were observed in the foetuses, but occassional capsular or cortical petechiae or ecchymoses were seen in lambs and adults. The organs may be congested and odematous, with capsules peeling with ease (Coetzer, 1977; Ikegami & Makino, 2011; Odendaal et al., 2019, 2020a, 2020b).

iv). Lungs and thoracic cavity

Foetuses: Haemorrhages were occasionally observed and serosanguineous effusions were common in the thoracic cavity (Odendaal et al., 2020b).

Lambs: Discernible oedema and congestion associated with mild to moderate hydrothorax were common (Odendaal et al., 2020a).

Adults: Oedema and congestion, with copious amounts of foam in the trachea and bronchi, accompanied by mild to moderate hydrothorax were a regular feature (Odendaal et al., 2019).

v). Heart

Foetuses: Gross lesions were rare but a case of subepicardial haemorrhage was reported (Odendaal et al., 2020b).

Lambs: Epicardial and endocardial haemorrhages as well as mild to moderate hydropericardium were common findings (Odendaal et al., 2020a).

Adults: Epicardial and endocardial haemorrhages and mild to moderate hydropericardium were common. Sub-epicardial ecchymoses were observed in the coronary grooves and were sometimes also present in paraconal and subsinuosal grooves. The left ventricle presented with sub-endocardial haemorrhages mostly (Odendaal et al., 2019).

vi). Lymph nodes and Thymus

Lymph nodes, especially those in the mesentry, were enlarged, oedematous, and congested in both lambs and adults. Petechiae or ecchymoses occurred in the thymus of lambs (Odendaal et al., 2019, 2020a).

vii). Adrenal gland

No lesions were seen in foetuses and lambs, but occassionally, cortical petechiae were observed in adults (Odendaal et al., 2019; Odendaal et al., 2020a, 2020b).

viii). Gastrointestinal tract and abdominal cavity

Foetuses: The abomasum presented with blood or blood-stained mucoid fluid and serosanguineous effusions were often present in the peritoneal cavity (Ondendaal et al., 2020b).

Lambs: Peritoneal and abomasal mucosal haemorrhages were seen (Odendaal et al., 2020a).

Adults: Multifocal serosal haemorrhages were present along the gastrointestinal tract, especially on the surfaces of the rumen and abomasum. Haemorrhages in the mucosa and submucosa, peritoneal hemorrhages along the major abdominal blood vessels, and mild to moderate serosanguinous ascites were observed in some adult sheep (Odendaal et al., 2019).

ix). Nervous and sensory systems

Mild to moderate oedema was seen in the central nervous system of foetuses and lambs (Odendaal et al., 2020a, 2020b). Foetuses of ewes inoculated with live-attenuated MP-12 vaccine strain at 28 to 56 days of gestation presented with cerebellar hypoplasia, spinal hypoplasia and hydranencephaly (Hunter et al., 2002). The cornea in 9 - 10-week old *Ripollesa* sheep which were subcutaneously inoculated with RVFV had corneal and choroidal oedema with inflammatory infiltrate (Busquets et al., 2010).

x). Skeletal and neuromuscular systems

Foetuses and lambs of pregnant ewes inoculated with live-attenuated MP-12 vaccine strain at 28 to 56 days of gestation presented with teratogenic effects such as prognathia inferior, brachygnathia inferior, arthrogryposis, scoiliosis, lordosis, kyphosis and domed heads (Hunter et al., 2002).

xi). Reproductive Organs

Adult testicles may present with multifocal petechiae to suffusive haemorrhages (Odendaal et al., 2019). The placenta presented with intercotyledonary oedema and the cotyledons were congested and necrotic (Odendaal et al., 2020b). Hydrops amnii was observed in pregnant ewes which were inoculated with live-attenuated Smithburn vaccine strain at 42 to 74 days of pregnancy (Coetzer & Barnard, 1977).

xii). Skin and Subcutis

Haemorrhages were common in the subcutis of lambs, while in adults they involved both subcutis and mucous membranes (Odendaal et al., 2019, 2020a).

b). Histopathology

i). Liver and Gall bladder

Foetuses: Necrosis of different severity was observed with no discernible zones except random hepatocyte necrosis or dropout of hepatocytes from the reticulin framework. Apoptotic bodies (Councilman bodies or acidophilic bodies), with condensed hypereosinophilic cytosol and marginalized chromatin, pyknosis or karyorrhexis, were often present. The architecture of the liver was largely intact in mild cases, with hepatocyte plates and sinusoids distinguishable with ease, but dropout of hepatocytes from the reticulin framework was also seen in these cases. Necrotic hepatocytes with karyolysis or karyorrhexis were sparse and randomly scattered throughout the liver parenchyma. Inflammation, if present, was mainly neutrophilic (Odendaal et al., 2020b).

Seemingly normal hepatocytes were present in clumps or short strings of up to 10 cells, with scattered interfering hepatocyte necrosis and hepatocyte dropout seen in moderate cases. The normal order of hepatocyte arrangement into plates was unclear, and sinusoids could not be distinguished. Neutrophils were often present while macrophages were absent (Odendaal et al., 2020b).

Severe cases were characterised by presence of small clumps or short strings of up to 5 surving hepatocytes with most nuclei in the majority of cells lost due to karyorrhexis or karyolysis, which was coupled with hepatocyte dropout and degenerate neutrophils. Complete dissociation of all hepatocytes and indistinguishable sinusoids and portal areas were infrequently observed. Haemorrhage or pooling of blood in areas depleted of hepatocytes were uncommon, whereas oedema was common in the portal area. Haematopoiesis was present in the majority of livers (Odendaal et al., 2020b).

Lambs: There was diffuse necrosis coupled with an infiltrate of degenerate neutrophils and few macrophages, usually associated with haemorrhage or severe congestion. Indiscriminately distributed, well-demarcated areas of liquefactive necrosis consisting of remnants of completely disintegrated hepatocytes, fine nuclear fragments, and a sparse infiltrate of degenerate neutrophils and macrophages in a collapsed reticular network, characterised primary necrotic foci. Few surviving hepatocytes showed degenerative (hydropic) changes. There was disruption of the hepatocyte arrangement and sinusoids were difficult to recognise. Hepatocytes with condensed hypereosinophilic cytosols and marginalized chromatin, pyknosis, or karyorrhexis were common. Some hepatocytes were swollen and rounded with disintegrated nuclei (karyolysis), or appear with small nuclear fragments (karyorrhexis). Collapsed hepatocytes due to rupture of the cell membrane were exhibited. Also necrotic were the limiting plate hepatocytes, and the connective tissue in the portal tracts was usually disrupted (Odendaal et al., 2020a).

Adults: Multifocal, focally extensive or confluent bridging necrotic foci which was not restricted to any particular area or zone, were found throughout the lobules, and normally accompanied by haemorrhage and mild to moderate infiltrate of neutrophils and macrophages. The portal tract interstitium was usually enlarged by mild oedema, with few mononuclear cells exhibiting some karyorrhexis. The bile ducts usually remained intact. Several hepatocytes exhibited apoptotic features such as dissociated cells, cellular shrinkage and rounding, hypereosinophilic cytoplasm, pyknosis and karyorrhexis, and fragmentation into multiple smaller apoptotic bodies. Varying degrees of micro- or macro-vesicular degeneration and anisokaryosis were present in adjoining hepatocytes. Adjacent sinusoids could contain scattered hyperplastic Kupffer cells. A clear presence of filamentous or oval eosinophilic intranuclear inclusions associated with nuclear chromatin marginalization in the hepatocytes were often observed (Odendaal et al., 2019).

The adult sheep gall bladder presented with haemorrhage in the serosa or adventitia, which could also be seen in the muscularis externa or lamina propria. Foci of necrosis could be seen in the lamina propria, serosa, or adventitia, with mild to moderate infiltrate of mononuclear cells and degenerate neutrophils. No lesions were observed in the foetal or lamb gall bladders (Odendaal et al., 2019, 2020a, 2020b).

ii). Spleen

Foetuses: Lymphocytolysis of varying degrees was a prominent feature. The periarteriolar lymphatic sheaths (PALS) consisted of a narrow zone of lymphocytes, and typical of foetuses, the follicular germinal centres and mantle zones were not well developed. Lymphocytolysis and occasional tingible-body macrophages were present in both the red and white pulp in the majority of spleens (Odendaal et al., 2020b).

Lambs: The spleen showed necrosis of varying degrees of severity, but the PALS appeared as a narrow zone of clearly identifiable cells. The follicular germinal centers, mantle cell layers, and marginal zones were mostly poorly developed due to young age. Necrosis thus mainly involved lymphocytes in the red pulp and the peripheral aspects of the PALS, but could be seen in both red and white pulp, and was characterised by clear presence of cellular debris and occasional tingiblebody macrophages (Odendaal et al., 2020a).

Adults: There were varying degrees of necrosis in the white and red pulp giving the samples a paucicellular appearance. Necrosis characterised by presence of cellular debris and tingible-body macrophages was apparent mostly in the germinal centers, mantle zones, marginal zones, and peripheral zones of the periarteriolar lymph sheaths (PALS) of the white pulp. The T-cell rich area of the PALS adjacent to the tunica media of the central artery did not show necrosis. The red pulp showed significantly less lymphocytolysis which occured as single cell necrosis (Odendaal et al., 2019).

iii). Kidney

Foetuses: Severe multifocal acute tubular epithelial injury without meaningful inflammation was observed in a few samples. Karyolysis of proximal tubular epithelial cells and presence of cellular debris and round desquamated cells with homogenous eosinophilic cytoplasm in the lumens of tubules, particularly in the superficial cortex and medullary rays, characterised the lesions. Haemorrhage was present in the cortical interstitium or below the capsule in a few cases. Glomeruli of lesser cellular density with pyknosis or karyolysis, and presence of nuclear debris in interstitial capillaries were observed in the majority of cases (Odendaal et al., 2020b).

Lambs: Severe multifocal acute tubular epithelial injury without significant inflammation was seen. Tubular epithelial cells showed degenerative changes such as fine vacuolation and swelling and impingement of the tubular lumen. The changes were sometimes coupled with pyknosis or karyolysis, with detachment of a few cells from the basement membrane. Marked acute renal tubular epithelial necrosis was not seen. The glomeruli appeared less densely cellular than normal with scattered pyknosis and karyorrhexis, and nuclear debris was also often present in the interstitial capillaries (Odendaal et al., 2020a).

Adults: Mild, moderate and severe multifocal acute tubular epithelial injury without significant inflammation was exhibited. Tubular injury was characterised by tubular epithelial cell pyknosis, karyorrhexis, and karyolysis, with detachment of these cells from the basement membrane. Infrequent haemorrhages could be seen in the glomeruli or the interstitium. Scattered nuclear pyknosis and karyorrhexis was present in the glomeruli and accompanied by occasional neutrophils. Similar to lambs, the glomeruli often appeared less densely cellular than normal, and interstitial capillaries presented with nuclear debris as well (Odendaal et al., 2019).

iv). Lung

Foetus: Different degrees of pulmonary interstitial oedema which expanded the connective tissue surrounding blood vessels, bronchi, or bronchioles and the pulmonary septa, was observed. Mild pyknosis and karyorrhexis were usually present in the alveolar septa and pulmonary blood vessels. Haemorrhage was present in the pulmonary septa or visceral pleura in several cases, and a few lung specimens were clearly aerated (Odendaal et al., 2020b).

Lambs: Intra-alveolar and interstitial oedema, and inflammation of varying severity in the alveolar capillaries consisting of mononuclear cells mainly, were seen. Pyknosis and karyorrhexis were often present in the alveolar septa and pulmonary blood vessels, and haemorrhage was seen in a few cases (Odendaal et al., 2020a).

Adults: Congestion, random haemorrhage, emphysema, and intra-alveolar and interstitial oedema were exhibited. Inflammation of varying severity, consisting of mononuclear cells and few neutrophils, could be seen in the alveolar capillaries. Single cell pyknosis and karyorrhexis occasionally occured in the alveolar septa and peribronchial lymphoid tissues, while haemorrhage was present in a few cases (Odendaal et al., 2019).

v). Heart

Foetuses and lambs: Lesions attributable to RVFV infection were not present in the cardiac parenchyma (Odendaal et al., 2020a, 2020b).

Adults: Focal haemorrhages in the endo- and epicardium were seen, but the myocardium normally did not show any RVFV infection lesions (Odendaal et al., 2019).

vi). Lymph nodes and thymus

Foetuses: Gross lesions could not be seen in the lymphnodes and thymus at necropsy (Odendaal et al., 2020b).

Lambs: Lymphocytolysis and lymphoid depletion of varying degrees was present. The follicular germinal centers were usually not well developed and apoptotic lymphocytes were scattered throughout the cortex and the medullary cords (Odendaal et al., 2020a). Petechiae or ecchymoses were seen in the interstitium of the thymus but lymphocytolysis as observed in other lymphoid organs was not present (Odendaal et al., 2020a).

Adults: Lymphoid necrosis characterised by varying degrees of follicular lymphocytolysis and cortical lymphoid depletion was present in many adults, sometimes with complete depletion of lymphocytes within the follicular germinal centers. Mild to moderate depletion of the mantle zone lymphocytes associated with dispersed lymphocytolysis in the interfollicular cortex and the paracortex was present. Depletion of the entire cortex is sometimes exhibited in severe cases with no distinction between the different parts *i.e* follicles, interfollicular cortex, and paracortex (Odendaal et al., 2019).

vii). Adrenal gland

Foetuses: No lesions were observed in the adrenal glands (Ondedaal et al., 2020b).

Lambs: Few necrotic cells were present in the zona fasciculata (Odendaal et al., 2020a). Adults: Lesions were characterised by multifocal adrenocortical necrosis and haemorrhage, with necrosis mainly involving individual, or cell aggregates in the zona fasciculate and zona glomerulosa to a lesser extent (Odendaal et al., 2019).

viii). Gastrointestinal tract and abdominal cavity

Foetuses: The lamina propria of the small intestine presented with foci of necrosis, with some cellular debris likely to have resulted from lymphocytolysis of the gut-associated lymphoid tissue (GALT). The connective tissue between the Peyer's patches showed karyorrhexis but the lymphocytes within the Peyer's patches were intact (Odendaal et al., 2020b).

Lambs: Blood was occasionally found in the lumen of the abomasum and the small intestine and necrotic foci were seen in the lamina propria of the small intestines. Karyorrhexis of cells in the lamina propria was common and probably contained lymphocytes, and karyorrhexis and pyknotic cellular debris were observed in the lymphoid follicles of the Peyer's patches (Odendaal et al., 2020a).

Adults: Necrosis was present in the lamina propria of the small intestines. Minute necrotic foci consisting of cellular debris and mild to moderate infiltrates of degenerate neutrophils and mononuclear cells were sparsely present between the intestinal crypts and at the base of the villi. Necrosis of the GALT and associated lymphocyte depleted Peyer's patches was observed. There may be nuclear and cellular debris in capillaries and connective tissue of the lamina propria.Submucosal haemorrhages may occur (Odendaal et al., 2019).

ix). Nervous and sensory systems

Foetuses and lambs: Central nervous system tissues presented mainly with mild to moderate oedema (Odendaal et al., 2020a, 2020b). Corneal and choroidal oedema with inflammatory infiltrate were observed in 9-10 week old Ripollesa sheep that were subcutaneously inoculated with RVFV (Busquets et al., 2010).

Adults: Congested and oedematous brain was observed by Coetzer (1977), while Olaleye et al. (1996b) reported mild gliosis, neural degeneration, neurophagia and satellitosis in the brains of RVFV infected sheep.

x). Reproductive system

Placenta: Oedema in the cotyledonary chorioallantois and necrosis of trophoblasts and endothelial cells in both the cotyledonary and intercotyledonary chorioallantois were prominently exhibited lesions. Diffuse necrosis of cotyledonary villi trophoblasts with multicellular debris between the villi was observed. Multifocal haemorrhages were common adjacent to the chorioallantoic villi, and were sometimes present in the myometrium or perimetrium. Fine basophilic granules were present in multiple single trophoblasts or in aggregates of trophoblasts in the chorioallantoic villi in several cases. Severe congestion and intravascular cellular debris were often seen, but necrotising vasculitis was infrequent (Odendaal et al., 2020b).

Adults: In male animals, haemorrhage was observed in the tunica albuginea and the connective tissue surrounding the seminiferous tubules, efferent ductules, and duct of the epididymis. Females displayed haemorrhages in the perimetrium and myometrium (Odendaal et al., 2019).

xi). Skin

There were no lesions observed in the minute number of foetuses and lambs examined (Odendaal et al., 2020a, 202b). Randomly distributed foci of haemorrhage and necrosis and multifocal cellular crusts overlying areas of mild eosinophilic inflammation were seen in some adult sheep, the later observation been probably due to insect byte hypersensitivity (Odendaal et al., 2019).

xii). Other organs

No gross pathology was observed for organs such as thyroid gland, pancreas, salivary glands, skeletal muscles and urinary bladder (Coetzer, 1977).

1.3.7 Cattle

a). Clinical signs

Signs of disease were mainly seen in the digestive and reproductive systems in cattle, in the form of salivation and foetid diarrhoea, and abortion and decreased milk production respectively (Adeyeye et al., 2011).

b). Gross pathology

i). Liver

The liver appeared swollen, orange-brown with greyish-white 0.5mm-1mm areas of necrosis, sometimes accompanied by congestion and haemorrhages (Coetzer, 1982). The gallbladder was usually oedematous and haemorrhagic.

ii). Other organs

Changes included enlarged and congested spleen, swollen, oedematous and haemorrhagic lymph nodes (cortex and medulla), congestion and oedema of the lungs, and haemorrhages on serosal surfaces of the internal organs. The abomasal folds were usually oedematous and haemorrhagic with fresh or digested blood in the lumen (Coetzer, 1982).

c). Histopathology

i). Liver

Centrizonal eosinophilic necrosis and haemorrhage affected the majority of the lobes, with karryorrhexic and pyknotic hepatic nuclei forming chromatin debris. The remaining hepatocytes were usually swollen, showing cloudy swelling, hydropic degeneration and fatty metamorphosis, with

acidophilic bodies found in both necrotic and degenerative hepatocytes (Coetzer, 1982). The changes in aborted foetuses and neonates were more severe than in adults.

ii). Other organs

Coetzer (1982) reported nephrosis characterised by tubular degeneration and necrosis of the glomeruli with proteinaceous fluid in the tufts, Bowman's spaces and lumen of the tubules. Pyknosis and karyorrhexis of the cell nuclei were seen. Single cell necrosis of the adrenal cortex was occasionally observed. Congestion, oedema, haemorrhage and emphysema were observed in the lungs. Disseminated serosal and submucosal haemorrhages of the small intestine and abomasum were common.

1.3.8 Goats

a). Gross pathology

Lesions observed by investigators were: i) pale liver with pinpoint red mottling on the anterior and posterior surfaces; ii) pale kidneys with sub-capsular haemorrhages and visible cortical striations; iii) normal-sized spleens with pinpoint haemorrhages below the capsule, along the periphery; iv) engorged mesenteric blood vessels, haemorrhages on the mesentery and enlarged and haemorrhagic mesenteric lymph node lymph nodes; and v) congestion of the ileum and testicles (Easterday et al., 1962).

b) Histopathology

i). Liver

Focal areas of necrosis with macrophage, lymphocyte and plasma cell infiltration disseminated throughout the lobules, and degeneration and necrosis of other parts of the liver (paracentral necrosis). Subcapsular hepatocytes appeared swollen degenerated and contained intranuclear inclusion bodies, while diffuse vacuolar degeneration of hepatocytes with Kupffer cells activation was observed in kids 2 weeks post vaccination with a Smithburn strain of RVFV (Ahmed Kamal, 2009). Massive destruction of the epithelial lining of the bile ducts with infiltration of lymphocytes and macrophages was seen.

ii). Other organs

Ahmed Kamal (2009) reported that spleen and lymph nodes showed hyperplastic activation of lymphocytes in the white pulp and paracortical zone respectively, with presence of intranuclear inclusion bodies in kids. Degenerative and necrotic changes occured in the proximal convoluted and distal tubules of the kidneys, with intranuclear inclusion bodies in the tubular epithelium observed. Congestion of the myocardium and blood vessels of the brain were seen.

The results of the above studies conducted on small mammals, humans, non-human primates and domestic ruminants clearly indicate that RVFV has a predilection for the hepatic tissue, and that the spleen, kidneys and neuronal tissues can also demonstrate macro- and microscopic lesions of RVF. For small mammals, it appears that mice, unlike rats and gerbils, are animals of choice for primary *in vivo* RVF laboratory work. Humans are clearly susceptible to RVFV and among the nonhuman primates studied, the rhesus macaque monkey seems to be a better model to study the disease in primates, in the absence of a better susceptible model that anatomically and physiologically resembles humans. Infection in sheep epitomises RVF in susceptible target ruminant species and the species remains the ultimate model for RVFV challenge studies.

1.3.9 Pigs

Gross pathology only revealed an enlarged lymph node on the side of inoculation of an experimentally infected weaner. Histopathology showed prominent follicular activity, mild lymphoplasmacytic perivascular cuffing and multifocal glial nodules with vacuolation of the neuropil in the brain (Clarke et al., 2021).

1.4 Rift Valley Fever Virus

Rift Valley fever virus is classified in the Bunyavirales order, *Phenuiviridae* family and *Phlebovirus* genus (Maes et al., 2018).

1.4.1 The virion

RVFV particles are icosahedral in shape, enveloped and measure approximately 90–110 nm in diameter (Freiberg et al., 2008) (Fig. 1.1). The envelope is composed of a lipid bilayer containing the G_n and G_c glycoproteins forming surface sub-units of approximately 5–8 nm in length, which are regularly arranged on its surface. Cryo-electron microscopy studies further revealed that not only is the virion structure highly ordered, but the surface is covered by a shell of 120–122 glycoprotein capsomers arranged in an icosahedral lattice with T = 12 (Pepin et al., 2010). The capsomers resemble hollow cylinders situated at five and six coordinated positions when viewed at 22 or 27 Å resolution. The viral ribonucleoproteins (RNP) corresponding to each of the three genomic segments and located proximal to the inner leaflet of the membrane, are packaged into the virion. There is no matrix protein in the Bunyaviruses.

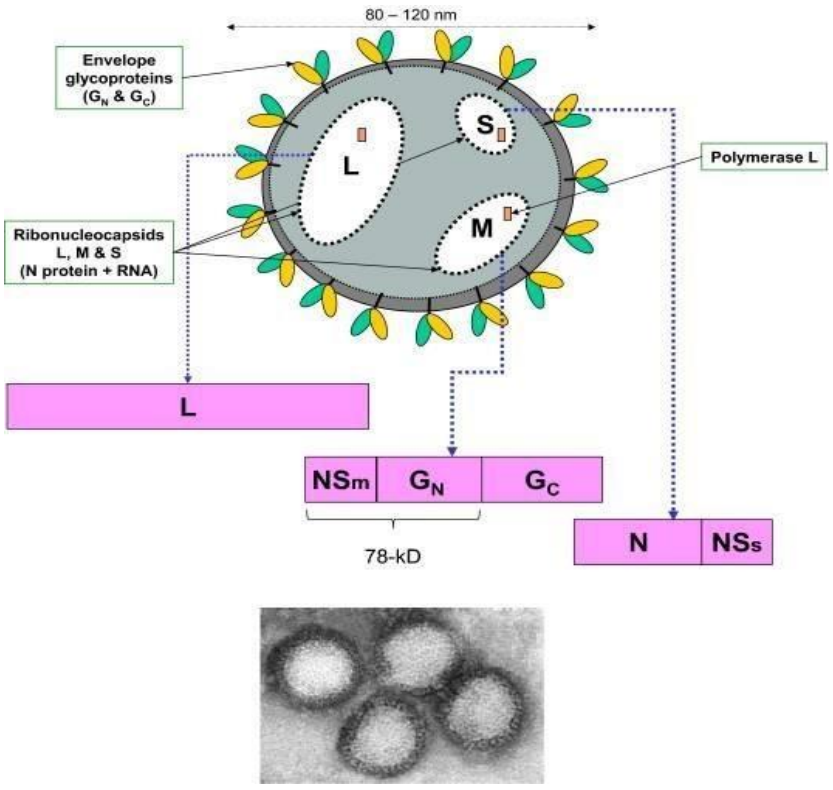

Figure 1.1 Schematic diagram of Rift Valley fever virus (electron micrograph from Linda Stannard), taken from Pepin et al., (2010). A colour version of this figure is available online at www.vetres.org

1.4.2 Genome

The genome of RVFV consists of a tri-segmented single-stranded RNA genome of negative polarity, namely, large (L), medium (M) and small (S) genome segments. The L-segment encodes the RNAdependent RNA polymerase (RdRp), which is responsible for transcription of genes and replication of the viral genome (Bird et al., 2009). The two major structural glycoproteins Gn and Gc involved in host cell entry and fusion, respectively, are coded for by the M-segment. Also coded by the Msegment are the two accessory proteins, NSm and a 78-kDa protein. The NSm have anti-apoptotic function (Won et al., 2007) and the 78-kDa protein is predominantly incorporated into virions matured in insect cells (Weingartl et al., 2014). Each genomic RNA segment is encapsidated by the nucleocapsid (N) protein to form a ribonucleoprotein (RNP) complex. The N-protein is coded by the –sense S-segment. In addition to the N-protein, the S-segment encodes the non-structural (NSs) protein which is regarded as the main virulence factor of the virus, and is known to antagonize host innate immune responses (Ly & Ikegami, 2016). The termini of all Bunyavirus genome segments are inverted complementary and facilitate the formation of a panhandle structure, which comprises signals for transcription, replication and encapsidation (Fig. 1.2).

1.4.3 Replication

Following exposure, RVFV virions use a C-type lectin expressed in immature dendritic cells (DCs) called DC-SIGN, as an entry receptor, and enter the cells (Terasaki & Makino, 2015). Successful entry into the cells is pH dependent and it is thought that the process may be a clathrin-mediated endocytic pathway described for another *Phlebovirus* (Bouloy & Weber, 2010; Ikegami & Makino, 2011). Inside the cell cytoplasm, the virus uncoats and the RNP complex (L, M and S RNA segments and N protein) is released into the cytoplasm, and the viral polymerase encoded by the L-segment initiates primary transcription to synthesize viral mRNA (Ikegami et al., 2005). During primary transcription, within the first hour after infection (approximately 40 minutes), the N and NSs mRNAs are transcribed from whole and well-packaged negative-sense and positive-sense S-segments, respectively. Within 1 to 2 hours post-infection (hpi), viral RNA replication commences, resulting in increased amounts of viral mRNAs and proteins. At 6-8 hpi genome segments are recruited to the Golgi, most probably through interactions between the N and cytoplasmic tail of Gn (WichgersSchreur & Kortekaas, 2016). Glycoproteins Gc and Gn are colocalised at the Golgi apparatus due to the motif targeting the Gc to the endoplasmic reticulum (ER), while Golgi retention signal retains the Gn at the Golgi. The cytoplasmic tail of Gn recruits the RNP and the RdRp for assembly, following which the progeny virions bud from the Golgi (Gerrard & Nichol, 2007; Gaudreault et al., 2019).

1.4.4 Physico-chemical characteristics and survival

a). Temperature

RVFV can survive in protein rich medium such as plasma and serum for up to 20 hours at room temperature (approximately 22°C), up to 8 months at 4°C – 5°C, 8 years under various and unspecified refrigeration conditions, but it cannot be recovered after 80 minutes at 56°C (EFSA, 2005; Daouam et al., 2014).

b). pH

The virus is most stable at pH ranges of 7.0 - 7.8, and labile at pH < 6.8 or > 8.0 (EFSA, 2005).

c). Precipitation and humidity

RVFV can be precipitated by ammonium sulphate, and survive in freeze-dried form and in aerosols at 23°C, under 50% – 85% of humidity (EFSA, 2005; OIE, 2019).

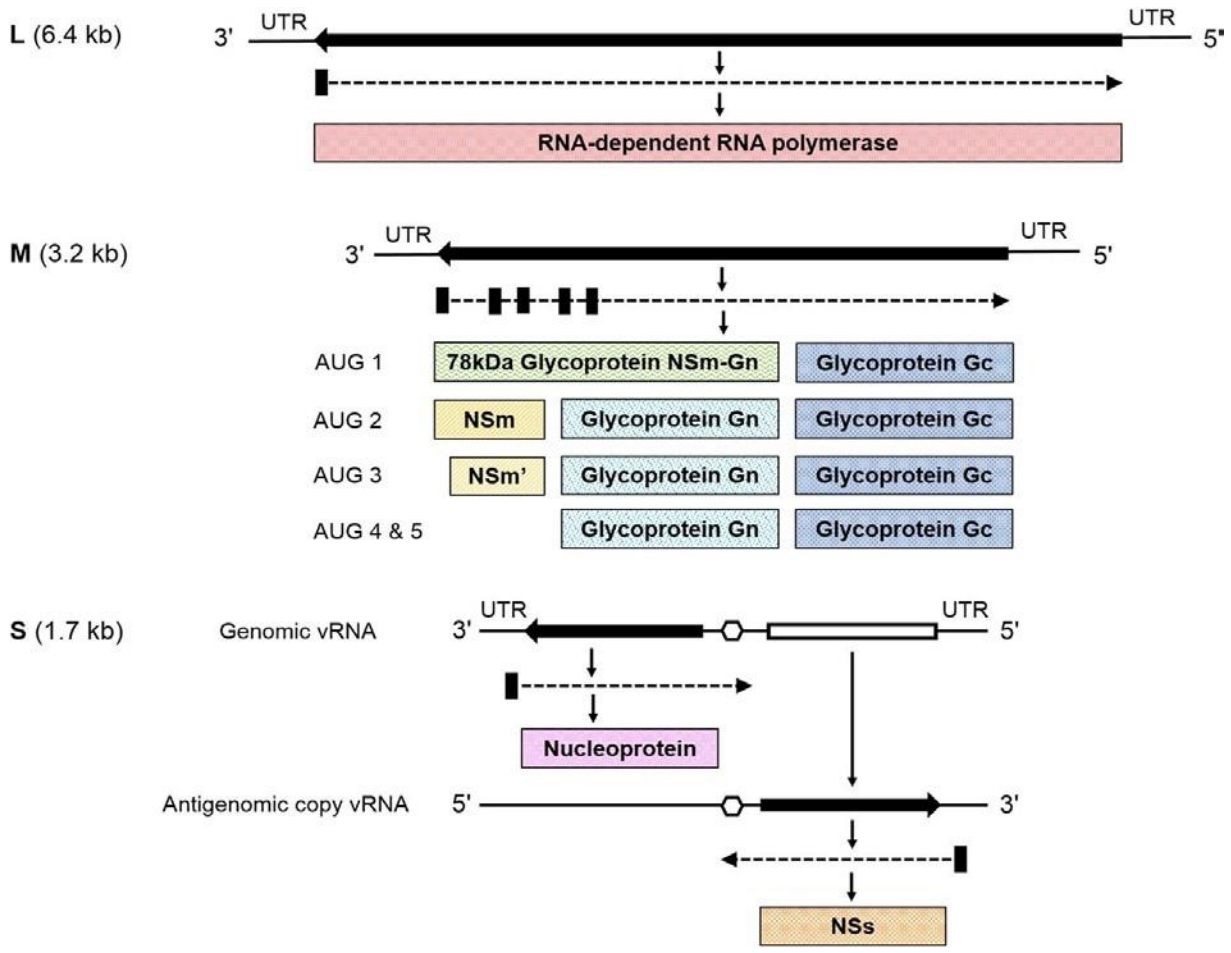

Figure 1.2 Linear representation of the genomic organization of Rift Valley fever virus, taken from Gaudreault et al. (2019). The encoded open reading frames are represented as solid black arrows on the large (L), medium (M), and small (S) segments of the viral genome. Untranslated regions (UTR) flank each segment. The length of each genomic segment is shown in kilo bases (kb). Transcribed messenger RNAs (mRNAs) are shown as dashed arrows with the start codons (AUG) represented as black boxes, and the resulting proteins shown as shaded boxes. Proteins encoded by the M segment are produced by posttranslational cleavage of translated polyproteins. An mRNA transcribed from the negative-sense genomic viral RNA (vRNA) generates the nucleoprotein. The non-structural S protein (NSs) is indicated on the genomic-sense S vRNA as a white box and is generated from an mRNA transcribed from the antigenomic copy vRNA produced during viral replication. The octagon indicates an intergenic transcription termination site

d). Disinfection

Virus particles are inactivated at pH <6.8. Disinfectants that can be used to inactivate the virus are lipid solvents such as ether, chloroform and sodium deoxycholate. Low concentrations of formalin and strong solutions of sodium or calcium hypochlorite (residual chlorine above 5000 ppm) can also be used to inactivate the virus. The virus can survive contact with 0.5% phenol at 4°C for 6 months (OIE, 2019).

e). Inactivation of samples in the laboratory

Research and diagnostic samples infected with RVFV can be de-activated using 4 % paraformaldehyde, Trizol LS, MagMAX™-96 Viral RNA Isolation Kit and Mag-Bind® Viral deoxyribonucleic acid (DNA)/ribonucleic acid (RNA) 96 Kit. The last three methods utilize chaotrope guanidinium thiocyanate to solubilise lipid bilayers and denature proteins, thus inactivating the samples (Bergren et al., 2020).

f). Natural survival

The eggs of *Aedes mcintoshi* mosquitoes have been proven to maintain RVFV during inter-epidemic periods (Linthicum et al., 2016).

1.5 Diagnosis

Rift valley fever is a zoonotic disease. Collection of suspect specimens for RVFV diagnosis should be carried out with utmost care, with consideration for sound biosafety and biosecurity regulations. The disease can be diagnosed by clinical observation and examination, and laboratory investigations that employ serological and agent identification methods. Depending on the epidemiological set-up, it is imperative that the correct assay, or combination of methods, be used for making a diagnosis (OIE, 2019).

1.5.1 Clinical signs

The clinical signs of RVF are not pathognomonic (fever, listlessness and inappetance) but the disease can be suspected when abortion storms and neonatal deaths in susceptible species are experienced following periods of heavy rainfall or flooding, and noticeable increases in the number of mosquitoes. Protective clothing should be worn when examining clinically affected animals and handling dead ones. The veterinarian can perform post mortem examinations and collect samples for laboratory analysis and confirmation of diagnosis (Davies & Martin, 2003).

1.5.2 Serology

The ideal sample for antibody detection is serum, even though some assays are robust enough to permit plasma. Live virus may be found in sera during viraemic stages of the disease and sera should be inactivated appropriately prior to testing.

The RVFV IgG and IgM antibody detecting ELISA and virus neutralization test (VNT) are the most popular serological methods in use for diagnostic purposes currently. Immunofluorescence assays are utilized to a lesser extent due to antigenic cross-reactions that usually occur between RVFV and other phleboviruses. Methods such as agar gel immunodiffusion (AGID), radioimmunoassays, haemagglutination inhibition (HAI), and complement fixation have been discontinued for RVF

serological diagnosis for reasons such as low sensitivity, specificity, reproducibility and robustness, and being laborious (OIE, 2019).

1.5.3 Agent identification methods

a). Virus isolation

Rift Valley fever virus can be isolated from serum, plasma or blood in viraemic animals, or liver, spleen, brain possibly other organs in carcasses, including aborted foetuses. Isolation can be done in cell cultures such as Vero (African green monkey kidney epithelial cells), BHK (baby hamster kidney), and Sf9 (*Spodoptera frugiperda* 9 insect cell line), or by intracerebral inoculation of sucking mice. Viral presence can also be shown by demonstration of presence of viral antigens or nucleic acid materials (OIE, 2019). Due to animal welfare and biosafety concerns, isolation in susceptible animals is generally forgone in favour of available *in-vitro* methods (OIE, 2019).

b). Reverse-transcription polymerase chain reaction (RT-PCR)

Unlike virus isolation, detection of viral RNA using validated conventional or real-time RT-PCR assays can yield results within hours of setting up the tests (Bird et al., 2007a, 2007b; Sall et al., 2001). RT-PCR methods for the detection of RVFV RNA can be applied to samples of human, vertebrate animal, and vector mosquito origin (Jupp et al., 2000).

c). Antigen detection immunochromatographic strip test and ELISA

An immunochromatographic strip test, commonly known as a lateral flow test (LFT) was recently developed for the detection of the nucleoprotein (NP) of RVFV (Cêtre-Sossah et al., 2019). The assay carries the advantage of not requiring specialized reagents and laboratory equipment to perform, making it an ideal first-line diagnostic tool for on-site RVFV detection in endemic countries. Antigen capture ELISAs such as the one developed by Fukushi et al. (2012)

d). Pathology

Macroscopic and microscopic examination of the animal or human body is an important diagnostic tool in disease investigations when determining the cause of death. Examination of cells, tissues and organs of affected animals can reveal cytopathology and lesions associated with a particular disease, and reveal the possible cause of death (Shieh et al., 2010).

i). Gross pathology

A full post mortem/autopsy is preferably performed on the day of death or following appropriate cold storage within three days. It involves careful examination of all organs and tissues involving all systems in the body in a systematic way. The position of the animal if found dead, position of the organs, colour, size, weight, shape, consistency, content, smell, distribution of lesions, and locality of the lesion on the particular organ are recorded. Samples of tissues, blood, other body fluids,

secretions and excretions, ingesta, and hair (and other keratinsed samples) may be collected in suitable containers and transport media for laboratory analysis (Otter & Davies, 2015).

ii). Histopathology

Preferred samples for histopathological examination are liver, spleen, kidneys and lungs since they have proven adequate for confirmation of diagnosis (Odendaal et al, 2019). A solution of 10% neutral buffered formaldehyde is commonly used as transport medium for pathology samples. Maintaining a cold chain is not a requirement, and transportation of the samples does not pose biosecurity risks as the formaldehyde inactivates infectious agents.

iii). Immunohistochemistry

Tissues fixed in formalin can be embedded in paraffin and further processed to identify viral antigens in antigen-antibody interaction based immunohistochemistry (IHC) tests (Odendaal et al, 2019; Taylor & Burns, 1974). The advantage of the method is that the colour signalled by the antigen– antibody complexes is visualized using light microscopy, morphology of the tissue around the specific antigen can be seen due to counter-staining, and results are reported semiquantitatively(Schacht & Kern, 2015).

1.6 Epidemiology

1.6.1 Vectors

Following the first documented reports of RVF in the Rift Valley of Kenya in the 1930s, endeavours to understand the disease, including methods of transmission, led to the identification of the mosquito as a primary vector of RVF (Daubney et al., 1931; Smithburn et al., 1949a). The vectors acquire infection from feeding on the blood of viraemic animals, and transmit the virus to other susceptible vertebrate hosts during subsequent feeding episodes. This mode of transmission is more common in animals, but human beings can also acquire infection through mosquito bites, sero-convert, and develop clinical signs of RVF (Laughlin et al., 1979; Gad et al., 1999; Sumaye et al., 2015).

However, not all mosquitoes are competent RVFV vectors. Presence of RVFV in several mosquito species and their vector competencies have been studied extensivelywith varying outcomes, from countries without infection and disease (Golnar et al., 2014; Iranpour et al., 2011; Klobucar et al., 2016; Linthicum et al., 2016; Moutailler et al., 2008; Turell & Kay, 1998), and those with infection but no reports of disease (Fontenille et al., 1998; Lee, 1979). The studies were also performed where outbreraks of RVF have been documented (Davies & Highton, 1980; Diallo et al., 2005; Fontenille et al., 1995, 1998; Gargan et al., 1988; Gear et al., 1955; Himeidan et al., 2014;

Hoogstraal et al., 1979; Jupp & Cornel, 1988; Jupp et al., 2002; Kokernot et al., 1957; Linthicum et al., 1985; Logan et al., 1991; McIntosh, 1972; McIntosh et al., 1980, 1983; Meegan et al., 1980; Ratovonjato et al., 2011; Smithburn et al., 1948; Taylor & Swanepoel, 980; Weinbren et al., 1957).

1.6.2 Horizontal transmission

Horizontal transmission of RVFV from one animal to another was demonstrated by experimental inoculation of various animal species with infectious materials using different routes (Table 1.1b - 1.1e). Daubney et al. (1931) successfully established infection *via* the intravenous route, while Findlay (1932a) infected animals utilizing several inoculation pathways *e.g* intraperitoneal, subcutaneous, intradermal, intracerebral, intravenous, intranasal and intra-tracheal. Easterday et al. (1962) also established infection intraperitoneally and intradermally, while Scott (1963) inoculated pigs intraperitoneally. Reed et al. (2013) and Hartman et al. (2014) demonstrated that infection could be established through inhalation of virus-containing aerosols in African Green monkeys and common marmosets.

Studies that sought to establish infection naturally through contact, ingestion and inhalation from infected to susceptible animals yielded conflicting results. Daubney et al. (1931) found that contact exposed lambs did not develop disease, and more recently, Wichgers Schreur et al. (2016) reported that immunocompetent or immunosuppressed lambs did not contract RVF when housed with RVFV infected lambs. Findlay (1932a) could not establish infection in mice and rats following feeding with livers and spleens from infected animals.

Successful horizontal transmission of RVFV was nonetheless reported by other workers. The virus could be isolated from saliva (Harrington et al., 1980; Busquets et al., 2010) suggesting the possibility of oral transmission. Transmission *via* the oral route was reported by Mims (1956) in mice which contracted the virus through cannibalism, and Easterday et al. (1962) who successfully infected lambs by swabbing their buccal mucosae.

Virus can also be isolated from nasal swabs, and infection *via* the respiratory route in mice, lambs and non-human primates was demonstrated by several investigators (Harrington et al., 1980; Easterday et al., 1962; Easterday, 1965; Busquets et al., 2010; Reed et al., 2013; Hartman et al., 2014). Contact transmission from infected mice to suckling ones was reported by Weiss (1957), and Busquets et al. (2010) also gave an account of one contact-exposed sheep.

The studies mentioned above indicate that even though possible, horizontal transmission of RVFV among animals in nature is not as efficient as that caused by vectors, and does not cause noticeable and sizable disease outbreaks (Wichgers Schreur et al., 2016).

In human beings, on the contrary, the respiratory system is the major route of RVFV transmission, where virus becomes aerosolized and inhaled during contact with and manipulation of infected carcasses and aborted foetuses. Laboratory personnel can also contract RVF through inhalation of virus at work when necessary precautions are not taken (Findlay, 1932a; Smithburn et al., 1949b; Mohamed et al., 2010; Archer et al., 2013). Sumaye et al. (2015) speculated that milkers could acquire infection through skin abrasions when milking viraemic animals, but this mode of transmission requires empirical evidence.

1.6.3 Vertical transmission

Researchers in RVF endemic areas observed that pregnant ruminants that suffered from the disease aborted, and RVFV could be isolated from their aborted foetuses and new-born offspring, often with the offspring showing characteristic hepatic lesions (Daubney et al., 1931; Coetzer, 1977; Coetzer et al., 1978; Coetzer & Schutte, 1978; Coetzer, 1980; Coetzer, 1982; Coetzer & Ishak, 1982; Coetzer et al., 1982; Van Der Lugt et a., 1996; Fafetine et al., 2016), proving that the virus can be vertically transmitted in vertebrate hosts.

Concerns of possible incursion of RVF into areas and regions free of the disease have led to various risk assessment studies aimed at assisting authorities with prevention strategies or control should the disease occur in their territories (Hartley et al., 2011; Golnar et al., 2014; Monaco et al., 2015; García -Bocanegra et al., 2016; Kim et al., 2016). Antonis et al. (2013) investigated the susceptibility of a breed of sheep, native to The Netherlands, to RVFV infection by inoculating pregnant ewes during the first, second, and third trimester of gestation. High mortality rates were observed among viraemic ewes, however, 4/11 inoculated ewes did not develop viraemia or clinical signs, did not have immunoglobulin G (IgG) or M (IgM) in their sera, yet RVFV RNA could be demonstrated in their organs and in those of their foetuses after death. The findings highlighted the fact that RVFV can be vertically transmitted in ewes in the absence of detectable maternal antibodies and viraemia, and that the virus may not be pathogenic in such situations. In additon to field and laboratory amplified viruses, RVF vaccine trails involving different vaccine formulations and doses, and domestic ruminants at different stages of pregnancy, proved that RVFV was vertically transmitted and that the virus could cause abortions and foetal malformations (Dungu et al., 2010; Hunter et al., 2002; Makoschey et al., 2016; Muller et al., 1995; Njenga et al., 2015). Vertical transmission of RVFV in humans was demonstrated

in Sudan and Saudi Arabia where infants born to RVFV infected mothers were born sick and died respectively (Adam & Karsany, 2008; Arishi et al., 2006).

1.6.4 World distribution

The geographic range of RVF was restricted to sub-Saharan Africa for approximately 88 years after it was first reported in 1931 (Daubney et al., 1931). Many African countries have infection and/or infection with disease, or serological evidence thereof. Figure 1-3 depicts the spatiotemporal distribution of the most notable outbreaks on the continent. Outside mainland Africa, RVF outbreaks were reported in the Comoros Island of Mayotte (1990 - 1991 and 2008) and the Republic of Madagascar (1990 -1991; 2008 - 2009). The disease was first diagnosed outside the African continent in the year 2000 and 2001, when it affected Saudi Arabia and Yemen (2000– 2001) (Nanyingi et al., 2015).

1.6.5 Inter-epidemic hosts and viral circulation

Whilst RVFV circulation generally occurs at 5 to 15 year intervals following periods of high rainfall, serological evidence of low levels of circulation among clinically and non-clinically susceptible species during inter-epidemic periods has been provided by numerous workers.

North Africa:

An inclusive seroprevalence of 2.29% was recorded during the inter-epidemic period of 2014 to 2015 in Egypt, among unvaccinated sheep, goats, buffalo and camels which were born in the country after the major outbreak of 2003 (Mroz et al., 2017). In Tunisia, a molecular and serological survey of febrile human patients and non-febrile agricultural and abattoir workers revealed 8.3% IgM and 7.8% IgG reactivity among the febrile patients and non-febrile workers, respectively (Bosworth et al., 2015). Also, Western Sahara which has never had outbreaks recorded a seroprevalence of 0.97% among its sheep, goats and camels (Di Nardo et al., 2014).

East Africa:

The last major RVF epidemics in East Africa occurred between 2006 and 2008, affecting Somalia (2006 - 2007), Kenya (2006 - 2007), Tanzania (2006 - 2007) and Sudan (2007 - 2008) (Himeidan et al., 2014). For determination of potential exposure to RVFV following the major disease outbreaks in Tanzania, humans, and sheep, goats and cattle which were present and not present during the 2006 - 2007 outbreaks were tested for RFV antibodies. Humans revealed a 9.8% seropositivity rate, while sero-prevalences of 22.7% and 5.5% were recorded for animals which were present during the outbreaks, and those which were born afterwards, respectively (Sumaye et al., 2013, 2015). In Kenya, wildlife and cattle were tested for anti-RVFV antibodies within the IEP spanning the years

2008 to 2015, and the overall seroprevalence was 11.6% in wildlife species while all cattle tested negative (Lwande et al., 2015). Zambia, a country which had not had RVF outbreaks in thirty years, conducted a similar study on sera from wildlife and domestic ruminants and other ungulates between 2014 and 2019. Wild ungulates had a seroprevalence of 33.7% compared to 5.6% for domestic ruminants (Chambaro et al., 2022).

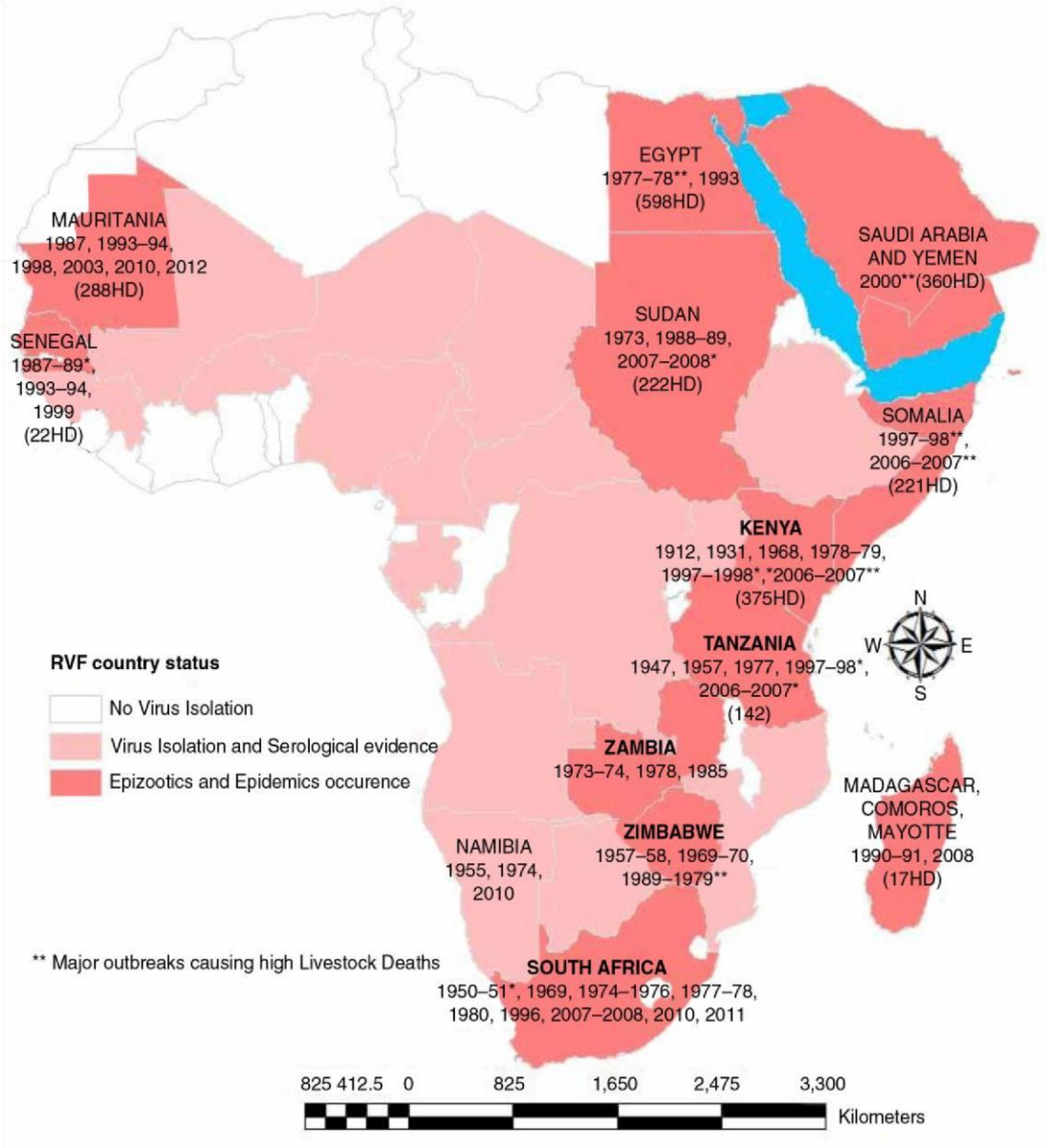

Figure 1.3 Spatial and temporal distribution of the Rift Valley Fever outbreaks affecting Africa and Arabian Peninsula from 1912-2012. Number of human deaths (HD) for all outbreak periods are indicated for some countries. Taken from Nanyingi et al. (2015)

Southern Africa:
Mozambique never reported clinical occurrence of RVF in at least 20 years prior to the 2014 outbreak. Serological surveys conducted in Zambezia Province in 2007 and 2010 involving sheep and goats revealed overall seropositivity rates of 35.8% in sheep and 21.2% in goats for 2007, and 9.2% in sheep and 11.6% in goats for 2010 respectively (Fafetine et al., 2013; 2016). In South Africa's Kruger National Park, African buffalo in 302 locations were tested for RVF sero-prevalence over a seven-year period (2000 - 2006) and 21% of them were found to be seropositive (LaBeaud et al., 2011). Fagbo et al. (2014) corroborated these findings by testing buffaloes from the Kruger National Park and Hluhluwe-iMfolozi Park during the IEP post the 2008 to 2011 epidemics and found 6.1% of the buffalos to be seropostive. Undetected virus continues to circulate in domestic ruminants, wildlife and humans in South Africa as attested by studies conducted in northern KwaZulu-Natal Province between 2016 and 2019. Seroprevalences of 34%, 31.7%, 35% and 2.8% were recorded for cattle, goats, nyala and impala, and humans respectively (Paweska et al., 2021; Van Den Bergh et al., 2019; 2020).

West Africa:
West African countries, Mauritania and Senegal, reported major outbreaks of RVF in 1987, and thereafter epizootics/epidemics of the disease followed in 1998, 2003 and 2010 in different provinces of Mauritania. A multi-stage serological and molecular analytical study among small ruminants, cattle and camels was performed by Rissmann et al. (2016) in Mauritania between 2012 and 2013 to determine RVFV activity in the absence of known outbreaks. A seroprevalence of 3.8%, 15.4% and 32.0% was observed for small ruminants, cattle and camels respectively. Studies conducted in sheep, goats and camels between March and April of 2008 in Western Sahara, Sahwari territories, showed an overall negligible RVF seroprevalence of 0.97%, (Di Nardo et al., 2014). More recently in Nigeria, Oragwa et al. (2022) proved seroconversion of 19.9% from human sera collected in 2019 from three states, without presence of disease.

Central Africa:
There is a paucity of information with regards the RVF situation among humans and animals in Central African countries. However, RVF serological surveillance conducted in rural populations of Gabon revealed a general seroprevalence of 3.3%, providing evidence of circulation of RVFV in the country (Pourrut et al., 2010). In Chad, two RVFV isolations were made from blood samples of two French soldiers who were among people who developed a self-limiting febrile illness between August and September 2001(Durand et al., 2003). In a subsequent study, Ringot et al. (2004) determined the seroprevalence of RVF in sheep, goats and cattle samples collected during the 2002 rainy season in Chad and recorded an overall seropositivity of 8.86% and 3.6% for IgG and IgM

respectively, indicating active circulation of RVFV in the country despite the absence of clinical cases in animals. Recently in Cameroon, a seroprevalence of 5.2% among sheep and goats sampled at a slaughter house in 2020 was reported (Ebogo – Belobo et al., 2022), supporting the notion of active virus circulation in the absence of active outbreaks.

1.7 Economic losses

Outbreaks of RVF impact not only livestock producers, but affect other livestock value chains and related sectors of the economy as well, including public health (Swanepoel & Coetzer 2004; Pepin et al., 2010; Rich & Wanyoike 2010; Kimani et al., 2016).

Numerous publications reporting the occurrence of RVF in several countries highlight the fact that controlling the disease is a multi-disciplinary effort, involving the health and agricultural ministries mainly, the defence ministries, non-governmental organisations and other sectors. The publications, however, fall short of estimating the total costs incurred in such endeavours, if not completely omitting the financial aspects associated with the outbreaks and control thereof (Woods et al., 2002; Munyua et al., 2010; Nguku et al., 2010; de St. Maurice et al., 2016).

1.7.1 Public Health

Human casualties during RVF outbreaks can easily be missed since the clinical signs of RVF in human beings range from mild febrile illness to severe disease that may be haemorrhagic or affect the nervous system and lead to death (de St. Maurice et al., 2016; Boushab et al., 2016; Salem et al., 2016). Table 1.2 indicates the number of human cases and fatalities recorded by the World Health Organisation (WHO) and other investigators from some of the most notable RVF outbreaks since the year 1990.

Economic losses associated with human RVF cases are due to loss of productivity when the patient dies or is sick and recuperating, cost of medication for supportive treatment, and implementation of control measures to prevent increases in disease incidence. Health economists use the Disability adjusted life years (DALYs), a non-monetary measure recommended by World Health Organisation (WHO), which reflects premature death and reduced quality of human life (disability) in non-fatal cases, to determine monetary and nonmonetary burdens of disease, including control. One DALY is equal to one lost year of “healthy life” (Murray, 1994).

Cost-effectiveness analysis (CAE) of disease burdens, expressed as cost of intervention per DALY prevented, assists authorities in determining existing or potential financial costs and gains of outbreak intervention strategies. Interventions that cost less than three times the national annual per

capita gross domestic product (GDP) are considered cost-effective, whereas those that cost less than one times the national annual per capita GDP are considered highly costeffective and ideal by the World Health Organisation (Marseille et al., 2015).

In the absence of financial reports articulating the financial burdens suffered by the public health sector due to RVF outbreaks, simulation studies incorporating the DALY concept in the models can be applied to estimate the costs of previous and predicted outbreaks. One such study was conducted by Kimani et al. (2016) in which the public health benefits of RVF control in livestock were assessed using the 2006/2007 and predicted 2014/2015 outbreaks in Kenya. The results showed that in a hotspot population of 2 657 137, there were 3974.05 DALYs, equivalent to 1.50 DALYs per 1000 people during the 2006/2007 outbreaks. The number of human casualties and deaths in the predicted 2014/2015 outbreaks was 158,525 and 78 respectively.

Schelling and Kimani (2008) and Orinde (2012) reported that Kenyan households spent approximately US$ 109.6–122.4 on hospital-related costs, while an additional US$ 70.8 per patient was footed by public hospitals on diagnosis, drugs and protective clothing. Kimani et al. (2016)'s simulation model predicted that the hypothetical 2014/2015 epidemic would result in 864 hospitalisations, with households bearing 63.4% of all costs incurred. Unfortunately, the model omitted direct recurrent costs such as deployment of additional personnel and staff salary per time spent on case management and surveillance. For the 2006/2007 epidemic however, the Kenyan government allocated US$ 1.3 million, and Non-Governmental Organisations (NGOs) provided an undisclosed amount of money for the outbreak response activities *e.g.* case management, community education, vector control and provision of mosquito nets, sampling and transportation of samples, laboratory diagnosis, surveillance and referrals of suspect cases (Kimani et al., 2016).

Table 1.2 Human casualties and fatalities recorded in some RVF outbreaks (1990 – 2016)

Country	Period of outbreak	No. of suspect cases (No. of confirmed cases)	No. of deaths	Reference
Kenya, Somalia and Tanzania)	1997 - 1998	89 000 (?)	478	Baba et al. (2016)
Saudi Arabia	October 2000	443	124	WHO, 2018
Yemen	October 2000	1328	166	Al-Afaleq and Hussein (2011)
Egypt	2003	45	17	Sayed-Ahmed et al. (2015)
Kenya	2006 –2007	75 000 (700)	158	Baba et al. (2016)

Country	Period of outbreak	No. of suspect cases (No. of confirmed cases)	No. of deaths	Reference
Somalia	November 2006 - 2007 February	30 000 (114)	51	Baba et al. (2016); Al-Afaleq and Hussein (2011)
Tanzania	2006 - 2007	40 000 (309)	144	Baba et al. (2016)
Sudan	November 2007 - 2008 January	75 000 (747)	698	Baba et al. (2016); Hassan et al. (2011)
Madagascar	2008	?(476)	19	Baba et al. (2016)
South Africa	2010 - 2011	? (278)	25	Jansen Van Vuuren et al. (2015)
Mauritania	September - October 2012	36	18	WHO, 2018
Senegal	September 2013 - 2014 February	535 (11)	0	Sow et al. (2016)
Uganda	March 2016	2(2)	2	de St. Maurice et al. (2016)
China	July 2016	1	0	Liu et al. (2017)
Niger	August – September 2016	105	28	WHO, 2018

?: Unknown/Not mentioned

1.7.2 Animal Lives

Similar to the human health situation, the full financial losses associated with RVF outbreaks and control in the agricultural sectors of affected countries are not, or, only partially reported (Sinyangwe, 2013; Mdlulwa & Ngwane, 2017; Muga et al., 2015). In animals, RVF manifests itself as a production problem, with abortion storms, death of neonatal animals, reduction in milk production, deterioration of animal health and physical condition (Mdlulwa & Ngwane, 2017; Muga et al., 2015). The disease can decimate three quarters of the total animal population, as reported in Garissa, Kenya, following the 2007 outbreaks, where farmers claimed that the disease was responsible for the deaths of 75% of their entire goat population, and a substantial number of cattle (Kenyan Department of Veterinary Services, 2007). For the 1997/1998 outbreaks, Kenyan livestock owners reported losses of approximately 70% of their sheep and goats, and 20% and 30% of their cattle and camels respectively (Muga et al., 2015). Table 1.3 indicates the number of RVF animal casualties and fatalities recorded by the Office International des Epizooties (OIE) and other workers since 1990.

In addition to the direct financial losses caused by deaths, abortions and reduced milk production, the cost of protecting susceptible animals at risk of contracting the disease through institution and application of zoo-sanitary measures and medical prophylaxis are substantial. It is estimated that direct and indirect losses suffered in Saudi Arabia due to the RVF outbreak in 2000 reached US

$75–90 million in one year (Hassan et al., 2014). During the 2006 - 2007 outbreaks in Kenya, at least 2,214,100 doses of a live attenuated vaccine were used. Insecticides were applied to almost all cattle, sheep, goats and camels in the affected districts, and antibiotics were procured for the symptomatic treatment and immune boosting of sick animals. Different investigators reported that the 2006 - 2007 Kenyan outbreaks cost the government between Ksh 2.1 and 4 billion (US$32 - 64.million) (Rich & Wanyoike, 2010; Muga et al., 2015).

Mdlulwa and Ngwane (2017) studied the socio–economic impact of the 2008 – 2010 RVF outbreaks in South Africa, in 2 municipalities of each of the 3 most affected provinces (Eastern Cape, Northern Cape and Free State), involving 150 livestock farmers. Livestock encompassed cattle, goats or sheep. Farmers indicated that they incurred extra expenditures because of the outbreaks, in the form of prevention (vaccination), control (dipping) and treatment (Terramycin to stimulate the immune system). It should, however, be noted that only half of them bought the vaccines from their own coffers, while the government supplied the vaccine to the remaining half. Approximately R480 000 (61 000 in 2014 Canadian dollar - C$) was spent on vaccinations, R2 million (240 000 in 2014 C$) on dipping and R18 000 (2 000 in 2014 C$) on Terramycin over the 3 year period.

The farmers in the survey reported 4 783 animal deaths and 6 460 abortions. Production losses stemming from mortalities, abortions and reduced milk production were estimated at R296 000 in 2008 (36,000 in 2014 C$), R480 000 in 2009 (59 000 in 2014 C$), and R4.4 million in 2010 (540 000 in 2014 C$). Of the surveyed farms, production losses of R1.8 million (220 000 in 2014 C$), 2.5 million (300 000 in 2014 C$), and R149 000 (18 000 in 2014C$) were recorded for the Eastern Cape, Northern Cape and Free State in the 2010 outbreaks, respectively (Mdlulwa & Ngwane, 2017). Mdlulwa and Ngwane (2017) employed the model they used in their study to estimate the total national production loss in 2010, and came up with a figure of R213.6 million (26.1 million in 2014 C$). The losses fed into the model included deaths of pregnant ewes/cows, deaths of non-pregnant ewes/cows, deaths of suckling animals, abortions, and reduction in milk production.

Factors due to other losses such as production of hides, wool and mohair, labour of draft animals, reduced market prices, trade restrictions and wedding dowries, were excluded.

Table 1.3 Select animal cases and fatalities recorded in the RVF outbreaks of 1990 - 2014

Country	Period of outbreak	No. of suspect cases (No. of Confirmed cases)	No. of deaths	Reference
Ethiopia, Kenya, Somalia, Sudan and Tanzania	1997 - 1998	?(?)	100 000	Anyamba et al. (2009)
Saudi Arabia	2000	10 000 (?)	40 000	Sayed-Ahmed and Nomier (2015)
Kenya*	Dec 2006 - July 2007	167 955 (?)	421 064	Rich and Wanyoike (2010)
Tanzania#	Dec 2006 - July 2007	19482 (?)	5 317 496	Chengula et al.(2013)
South Africa^	2010 - 2011	16000 (?)	12400	Monaco et al. (2013); South African Government, 2011; Glancey et al. (2015)
Senegal	September 2013 February 2014	492 (52)	11	Sow et al.(2016)

*: Figures for 2 districts; #: Figures for 3 regions only; ?: Unknown/Not mentioned

1.7.3 Trade

Business is negatively affected during and in the aftermath of RVF outbreaks. The disease is listed by the OIE due to its effects on animal and human health, and potential for rapid transboundary spread (OIE, 2018; OIE, 2019). Trade restrictions on susceptible animals and their products are instituted by importing countries when exporting countries experience occurrences of listed diseases (Kasye et al., 2016). Such bans are usually maintained until the affected countries can provide scientific proof of their regained freedom from disease.

Trade within the borders of the affected country is also debilitated by control measures implemented during the outbreaks, in the form of farm quarantine, movement restrictions, and closure of abattoirs and market places. Livestock prices before the onset of outbreaks, and during and after, are usually significantly different. Prices befitting the value of the animals are obtained before the outbreaks, but, a reduction is experienced during outbreaks due to declined demand, closure of markets, suboptimum condition scores of the animals due to illness or inability to travel to lush pastures, movement restrictions and quarantines.

There is a shortage of information regarding the actual tariffs lost due to bans on exports from many countries and regions, but experiences of Ethiopia, Kenya, Somalia and Saudi Arabia between 1998 and 2007 provide an indication of financial losses suffered. The first (February 1998 – May 1999) and second (September 2000 – December 2002) livestock export bans by Arab countries due to the RVF outbreaks that occurred in the Horn of Africa between 1997 and 2001 caused the Republic of Somaliland US$109 million and US$326 million respectively. The losses were a result of an estimated amount of 8.2 million small ruminants, 110 000 camels and 57 000 cattle that could not be exported (Wanyoike et al., 2019). The Somali region of Ethiopia lost an estimated income of US$132 million from the imposed embargo on exports to the Gulf in 2000 (Rich & Wanyoike, 2010). Trade of live animals within Saudi Arabia estimated to be worth US$ 600–900 million annually was also affected by the ban of livestock importation from East African countries, thus, the bans severely crippled both the exporters and the importer (Hassan et al., 2014). For the 2006-2007 outbreaks in Kenya, more than US$9.3 million was lost due to a ban on livestock trade and imposition of quarantine (Muga et al., 2015).

1.8 Prevention and Control

Due to its zoonotic nature, a “One health approach” involving a wide range of institutions and authorities with different expertise is advocated for responding to RVF outbreaks (Alam & Mohammed, 2000; Fawzy & Helmy, 2019). The following activities would be conducted during IEPs and in the phase of an outbreak:

1.8.1 Prevention

a). Sentinel Herds

Sentinel animals are an important part of disease surveillance, and provide baseline epidemiological information with regard to inter-epidemic viral activity, identify areas at risk, give warning about increased virus activity, and serve as early indicators of emergence of RVF outbreaks. Countries such as Kenya, Mauritania, Saudi Arabia and Senegal have used sentinel herds to detect RVFVinfected mosquito activities at strategic locations (Lancelot et al., 1990; Zeller et al., 1997; Al-Qabati & Al-Afaleq, 2010; Lichoti et al., 2014).

Sentinel animals should ideally number approximately 30, and should be 12-15 months old, they should be RVFV antibody negative susceptible ruminants, preferably female, so that they can be retained longer for breeding purposes, and placed in geographically representative areas where mosquito breeding activity is likely to be the highest. Acaricides, pour-ons or insecticides should not be applied on the animals so that the mosquito attacks cannot be interfered with. The sentinel herds

should be sampled at the beginning of the rainy season and thereafter every four to six weeks until the end of the rainy season, and their sera tested for presence of RVFV IgM and IgG antibodies. All activities should be coupled with clinical monitoring (Davies & Martin, 2003).

b). Early warning: Satellite imagery

The link between RVF epizootics and El Niño/Southern Oscillation (ENSO) phenomena, which is characterised by episodic anomalous warming and cooling of sea surface temperatures (SSTs), was confirmed following the 1997–1998 epizootic/epidemic in the Horn of Africa (HOA) (Anyamba et al., 2001; Anyamba et al., 2010). Abnormally warm SSTs in the equatorial eastern central Pacific Ocean region and the western equatorial Indian Ocean result in above-normal and widespread rainfall in the HOA, increased populations of infected mosquito vectors, and RVF epizootics/epidemics (Linthicum et al., 1999).

Researchers analyse El Niño/Southern Oscillation related climate anomalies by employing a combination of satellite derived measurements of elevated SSTs, resultant increased rainfalls and satellite-derived normalised difference vegetation index data. The data can be used to compute disease risk assessment models, as was done for RVF in the HOA (Anyamba et al., 2001; 2009; Kovats et al., 2003).

The spatio-temporal occurrence of the 2006-2007, 2006-2008 and 2008 RVF epizootics/epidemics in the HOA, Sudan and South Africa respectively, were predicted using climate data and provided a 2 to 6 week period of preparation of outbreak response strategies and accumulation of essential resources. Entomological and epidemiological field investigations in the aftermath of the outbreaks corroborated the accuracy of the predictions in the areas identified as being at risk (Anyamba et al., 2009, 2010).

c). Vector control

Preventing mosquitoes from feeding on animals and humans, and transmitting RVFV is ideal, but unrealistic following periods of high rainfall, when increases in the number of these vectors are experienced. Insecticides, chemical or microbiological larvicides and ovicides, pupicides, hormonal inhibitors and larvical toxins, phytochemicals and nets have been used to prevent bites or control mosquito populations nonetheless (Alam & Mohammed, 2000; Benelli, 2016; Chowdhury et al., 2008; Haldar et al., 2011; Mahgoub et al., 2017; Rawani et al., 2017; Tran et al., 2016).

d). Vaccination

There are four types of licensed RVF veterinary vaccines for use in the protection of domestic ruminants available, and none for humans. These are: i) the live attenuated (Smithburn); ii) inactivated (Smithburn); iii) live attenuated (MP-12); and iv) live attenuated (Clone-13) (Dungu,

Lubisi, & Ikegami, 2018; Mansfield et al., 2015). Despite the fact that these vaccines have proven to be efficacious in the prevention of RVF in ruminants, there is emperical evidence which suggests that the live attentuated formulations may not be totally safe when used under certain physiological and epidemiological conditions. The quest for safer, efficacious, and cheaper veterinary and human vaccines for use under different conditions thus continues. Tables 1.4a to 1.4b summarises important characteristics of the licensed veterinary vaccines, and their advantages and disadvantages.

e). Movement restriction

The restriction of movement is usually indicated when importing animals from an area with infection alone, or infection with disease, until conditions such as those stipulated in the OIE Terrestrial Animal Health Code (2018) are met. This is because there is a real risk of introduction of RVF into an infection or disease free area if incubating viraemic animals arrive in an area with a large number of competent mosquito vectors which could spread the virus. RVFV-infected mosquitoes can cause an outbreak in a RVF-free area (Chevalier et al., 2010; Hartley et al., 2011; Samy et al., 2017).

1.8.2 Control

a). Disposal of carcasses

Personal protective equipment (PPE) such as gloves, protective eyewear, overalls, rubber boots, and respirators should be worn and used by people handling aborted foetuses, carcasses or milking potentially infected animals. Carcasses should be burnt or buried in deep holes and covered with a layer of soda lime or other recommended potent chemicals to disinfect the virus on the surfaces. Prior to disinfection of infected premises, animal enclosures, utensils and equipment that have come into contact with infected animals and their body fluids should be cleaned, and organic materials such as dirt, manure and plant remains removed since many disinfectants are ineffective in the presence of such organic matter (Davies & Martin, 2003).

b).Quarantine and restriction of animal movement

Movement of RVFV susceptible animals from infected areas to non-infected locations during outbreaks is restricted, as was done in Saudi Arabia during the outbreaks of 2000. This is done to reduce the possibility of them acting as sources of virus for vectors at their destination, thus causing more outbreaks (Alam & Mohammed, 2000). Since human-to-human transmission of RVFV has not been demonstrated, isolation of RVFinfected or in-contact people is not necessary. No specific treatment, except for supportive treatment, is available for infected individuals. Recovered patients should be monitored for possible development of complications.

c). Vaccination in response to the outbreak (s)

Emergency vaccinations can be carried out to prevent spread of the disease in the early phase of an outbreak. This is normally done with inactivated vaccines to prevent development of viraemia

from live attenuated vaccines and possible virus uptake by mosquito vectors that would infect more susceptible animals and exacerbate the problem.The inactivated vaccine is also safe for use in pregnant animals (Dungu et al., 2013).

d). Control of vectors and parasites

Vector control during outbreaks is carried out in the same manner as when the technique is used as a preventative measure.

Table 1.4a List of licenced RVFV vaccines available for veterinary use, taken from Mansfield et al. (2015)

	Smithburn live attenuated virus vaccine	**Inactivated virus vaccines**	**Clone-13 live attenuated virus vaccine**	**MP-12 live attenuated virus vaccine**
Virus origin	Mosquito, Uganda, 1948	Field strains (South Africa and Egypt)	Human, Central African Republic, 1974	Human strain ZH548 (Egypt, 1977)
Attenuation	>200 passages in mouse brain	N/A	Natural deletion in NSs gene	Multiple mutations in S, M and L segments
	Smithburn live attenuated virus vaccine	**Inactivated virus vaccines**	**Clone-13 live attenuated virus vaccine**	**MP-12 live attenuated virus vaccine**
Production medium	BHK cells	BHK cells	Vero cells	MRC-5 cells
Adjuvant	None	Aluminium Hydroxide	None	No adjuvant
Form/Presentation	Lyophilised	Liquid	Lyophilised	Lyophilised
Regimen	Single dose - sheep and goats Double doses - Cattle	Requires booster and annual revaccination	Single dose	Single dose
Licensed	Yes	Yes	Yes (South Africa, Kenya and Zimbabwe)	Conditionally in the United States of America

N/A: Not applicable

Table 1.4b Advantages and disadvantages of the currently available RVFV vaccines

Vaccine	Advantages	Disadvantages	Reference
Inactivated	i. Safe in pregnant animals ii. Can be used during outbreaks iii. Ideal for use in low risk zones	i. Expensive ii. Booster dose is required iii. Mandatory annual re-vaccination iv. Not practical in routine vaccination programmes v. Has no attributes to be used for DIVA purposes	Alhaj, 2016; Faburay et al. (2017)
Live attenuated	i. Affordable ii. Single dose required per vaccination iii. Confers lifelong immunity	i. Abortogenic in pregnant ewes ii. Cause teratogenic effects iii. Associated with high viraemia iv. May revert to virulence v. Can only be used in endemic areas vi. No DIVA attributes	Alhaj, 2016; Faburay et al. (2017)
Clone 13	i. Affordable ii.Single dose sufficient iii. Confers long-term immunity iv. Safe in pregnant animals v. Associated with very low viraemia vi. Has DIVA potential	i. Short shelf-life ii. Risk of genetic reassortment iii. Restricted to endemic zones iv. Associated with malformations and still births in experimentally infected pregnant ewes when administered at higher doses than recommended, particularly in the first trimester	Alhaj, 2016; Faburay et al. (2017)
MP-12	i. Affordable ii.Single dose sufficient iii. Confers long-term immunity iv. Safe in pregnant animals v. Applicable for us in humans as well vi. Associated with very low viraemia vii. Has DIVA potential	i. Short shelf-life ii. Risk of genetic reassortment iii. Restricted to endemic zones iv. Possibility of malformations and still births in experimentally infected pregnant ewes in the first and second trimester	Faburay et al. (2017); Ikegami, 2017

1.9 Problem statement

RVF outbreaks result in devastating health consequences for both humans and livestock, and impact negatively on the economy of affected countries. Vaccination remains the only effective way of protecting livestock from the disease since vector control has proven impractical (Bird & Nichol, 2012). To facilitate outbreak response and mitigation activities, risk mapping, predictive models using satellites and climate data, and early warning systems (EWS) are currently employed in RVF-prone areas. However, effective EWS development with increased predictive accuracy requires both

climatic and non-climatic information (WHO, 2009). Non-climatic factors include indicators of the vulnerability of populations to disease outbreaks such as exposure to viruses, infection, immune response, spread, and adaptation.

Pigs are integral to Africa's mixed species farming systems, come into contact with humans, are bitten by mosquitoes, scavenge and eat dead animals including aborted foetuses, and have been proven to sero-convert following RVFV infection in Egypt (Wabacha et al., 2004; Youssef, 2009; Thomas et al., 2013). The studies conducted in Egypt provided supportive evidence of the potential role of this species in the epidemiology of the disease. It should be remembered that the two major obstacles for determining natural host ranges of viruses are incomplete field investigations and inability to segregate hosts essential for prolonged biological transmission from other susceptible vertebrates. The degrees of contribution to viral transmission are not the same among competent vertebrates (Kuno & Chang, 2005).

A recent review on the role of wild mammals in the maintenance of RVFV stated that data currently available were too minimal for conclusive proclamations to be made on the potential role of wild and domestic pigs in RVFV maintenance (Olive et al. 2012). The role of domestic pigs (*Sus scrofa*) in the epidemiology of RVF has not been thoroughly investigated, and research conducted in the 1950s to 1960s provided inconclusive and circumstantial evidence of porcine susceptibility to RVFV and conditions for successful infection (Scott, 1963). This has led to the general assumption that the species is refractory to RVFV infection. Knowledge gaps in the epidemiology of RVF with special reference to virus host range and susceptible species warrants further research on the subject, in the interest of devising improved control strategies.

1.10 Aim

The study aimed to use modern laboratory diagnostic methods to address the shortcomings of previous studies through: i). investigation of the outcome of RVFV infection in pregnant sows, neonatal piglets and weaners; ii). identification of the most suitable serological method to detect RVFV antibodies in the porcine species; and iii). determination of the prevalence of RVF antibodies in wild and domestic suid populations in South Africa. It is anticipated that these results would provide a better scientific basis to inform decisions on whether to include or exclude this species in nonclimatic EWS information in pig farming areas of Africa that experience atypical RVF outbreaks, or to use pigs in RVF biomedical research.

CHAPTER II

EXPERIMENTAL INFECTION OF DOMESTIC PIGS (*SUS SCROFA*) WITH RIFT VALLEY FEVER VIRUS

Abstract

Rift Valley fever (RVF) is a vector-borne viral zoonotic disease, which primarily affects domestic ruminants. It is characterised by abortion storms and neonatal deaths in animals, and self-limiting flu-like symptoms which can progress to more severe disease or death in humans. The susceptibility of domestic pigs (*Sus scrofa domesticus*) to RVF virus (RVFV) is largely unresolved due to conflicting experimental infection results. Additionally, limited information is available regarding the course of infection and clinico-pathological lesions, particularly in pregnant sows and neonates. To address this, we infected two groups of pregnant sows, neonates and weaners, each with a different RVFV isolate, and a third group of weaners with a mixture of the two viruses. Serum, blood and oral, nasal and rectal swabs were collected periodically, and two neonates and a weaner from groups 1 and 2 each, euthanised from 2 days post infection (DPI), and necropsy and histopathology samples were collected. The samples were tested for presence of RVFV antigen in organs displaying characteristic lesions, antibodies in sera, and RNA in organs, blood and pooled oronasorectal swabs. Positive results were obtained for several of the infected pigs. Results confirmed that pigs can be experimentally infected with RVFV, although sub-clinically, and showed that pregnant sows can abort following infection with high viral doses. Although the likelihood of natural infection with RVFV is low in pigs, the demonstrated presence of viral RNA in oronasorectal swab pools on 28 DPI suggest that pigs may shed RVFV for at least one month. We conclude that precautions similar to those taken with ruminants should be applied when supervising pig obstetric procedures and carcass disposal during RVF outbreaks, to prevent possible transmission of virus from this species to humans.

Keywords: Pathology; Polymerase Chain Reaction; Serology; Sequencing; Phylogenetics

2.1. Introduction

Rift valley fever (RVF), first described by Daubney et al. (1931), is a vector-borne zoonotic disease, which primarily affects domestic ruminants and camelids. It is caused by RVF virus (RVFV), in the Bunyavirales order, *Phenuiviridae* family and *Phlebovirus* genus (Maes et al., 2018). Animals are predominantly infected through the bite of infected mosquitoes, but vertical transmission is possible (Fafetine et al., 2016). Transmission to humans can occur through contact with aerosolised virus during handling and opening of infected carcasses, and to a lesser extent *via* mosquito bites. The disease is characterised by abortion storms and neonatal deaths in animals, while humans normally present with self-limiting flu-like signs. However, the disease can progress to severe hepatic disease with haemorrhagic manifestations, renal impairment, encephalitis, ocular complications and death (Kasye et al., 2016; Odendaal et al., 2020a; Odendaal et. al. 2021). Diagnosis of RVF employs antibody and antigen detection methods including virus isolation, virus neutralization, RT-PCR, ELISA or histopathology with immuohistochemistry (OIE, 2019). Outbreaks of RVF may have serious economic impacts due to imposed trade bans and devastating health consequences for both humans and livestock. Vaccination remains the only effective way of protecting livestock from the disease since vector control is impractical (Bird & Nichol, 2012).

Effective outbreak response and mitigation activities rely on vaccination, risk mapping, predictive models using satellites and climate data, and early warning systems (EWS) in RVF-prone areas (WHO, 2009). However, effective EWS development with increased predictive accuracy requires both climatic and non-climatic information (WHO, 2009). Non-climatic factors include indicators of the vulnerability of populations to disease outbreaks and include host exposure to viruses, susceptibility to infection, immune response and virus adaptation, shedding and spread. The two major obstacles for determining natural host ranges of viruses are incomplete field investigations and the inability to segregate hosts essential for prolonged biological transmission from other susceptible vertebrates. This is because the degrees of contribution to viral transmission are not the same among competent vertebrates (Kuno & Chang, 2005).

Pigs are integral to Africa's mixed species farming systems. They come into contact with humans, are bitten by mosquitoes, scavenge and eat dead animals including aborted foetuses, and have been shown to sero-convert following natural infection with RVFV based on studies conducted in Egypt (Wabacha et al., 2004; Youssef, 2009; Thomas et al., 2013). However, the role of domestic pigs (*Sus scrofa*) in the epidemiology of RVF has not been thoroughly investigated. Research conducted in the 1950s and 1960s provided conflicting and circumstantial evidence of porcine susceptibility to RVFV, (Scott, 1963), leading to the general assumption that the species was

refractory to RVFV infection. Scientists, however, concede that there was minimal information available for conclusive assertions to be made on domestic pig susceptibility and their potential role in RVFV maintenance (Clarke et al., 2021; Olive et al. 2012). Knowledge gaps in the epidemiology of RVF with special reference to pig susceptibility to RVFV led to a recent study in which weaners were successfully infected with the ZH501 strain of RVFV, as proven by seroconversion, and virus isolation from sera and oronasal swabs, and RNA isolation from the isolates (Clarke et al., 2021). These latest findings warrant additional efforts to establish the effects of RVFV on pig neonates and pregnant sows, in the interest of determining their potential role in virus maintenance and transmission, and if necessary, devising improved prevention and control strategies. This is particularly important in light of increasing urbanisation and swine populations in Sub-Saharan Africa (Penrith & Kivaria., in press).

Until the recent study by Clarke et al. (2021), early experiments to determine swine susceptibility to RVFV only involved innoculating fewer than five pigs of undisclosed ages and mostly measuring their temperatures and determining viraemia or antibody presence, without providing details of the tests used (Daubney et al., 1931; Easterday et al., 1962; Findlay & Daubney, 1931; Scott, 1963). The aim of the present study was to address shortcomings of previous studies through experimental infection of pregnant sows at different gestation periods, neonatal piglets of less than one week, and weaners. Ante and post mortem samples from the pigs were periodically collected, and analysed using serological and antigen detection assays for a period of sixty days. A futher aim was to inform decisions on whether to include or exclude this species in non-climatic EWS information in pig farming areas of Africa that experience atypical RVF outbreaks, thus clarifying their relevance in the epidemiology of the disease.

2.2 Materials and Methods

2.2.1 Virus strains and cell culture

Two RVF viruses isolated at the Agricultural Research Council – Onderstepoort Veterinary Research (ARC – OVR) were used in the challenge experiments. Virus 1 (a variant of M66/09) was a bovine liver isolate from the 2009 RVF outbreak in the Gauteng Province of South Africa, used at passage 5BHK.15Vero and a titre of $5X10^7$ pfu/ml. Virus 2 (a variant of M21/10) was an ovine organ pool isolate from the 2010 outbreak in the Free State Province of South Africa, used at passage 3BHK.14Vero and a titre of $1.5X10^5$ pfu/ml. The viruses were propagated in Vero cells (ATCC, USA) maintained with Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, USA) containing 2% foetal bovine serum (FBS) (Sigma-Aldrich, USA) and 1x Penicillin and Streptomycin each (Gibco, USA) and incubated at 37 °C with 5% CO_2. Partial nucleotide sequencing of the glycoprotein gene

(Gn) encoded by the M - genome segment was performed for the two viruses prior to serial passaging in organ culture for purposes of verifying their genotypes. Strains M66/09 and M21/10 belong to two different S, M and L segment genotypes (Maluleke et al., 2019).

2.2.2 Animals and Experimental Design

Animal experiments were performed following appropriate acclimatisation periods at a biological safety level 3 (BSL3) animal facility at ARC – OVR Transboundary Animal Diseases Programme (TADP), under animal ethics committee (AEC) approval numbers AEC10.16 and EC057-17. Large white pregnant sows (PS: n = 9), lactating sows (LS: n = 3), 1 - 3 day old suckling piglets (SP: n = 30), and 6 - 8 week old weaners (W: n = 27) were obtained from the ARC Animal Production Institute in Irene, Gauteng Province. Black head Dorper lambs (L: n = 8), 1 – 2 weeks old, and their dams, the ewes (E: n = 8), were sourced from a commercial farm in the Northern Cape Province. The animals were divided into three groups (1 - 3), and inoculated with virus 1, virus 2 and a mixture of both virus 1 and virus 2, respectively. Group 1 animals (n = 29) were housed in stables A and B, group 2 (n = 29) in stables C and D and group 3 (n = 17) in stable E. Accommodation, animal identities and treatment regimens are detailed in Appendix 1.

Two suckling piglets in stables A and C each, one weaner in stables B and D each, and two weaners in stable E served as mock inoculation controls and were infected with 2ml of tissue culture medium intravenously (i/v). No lactating animal was inoculated. All remaining animals in group 1 (stables A and B) and group 2 (stables C and D) were inoculated with 2ml of virus 1 (i/v) and virus 2 (i/v), respectively. In group 3 (stable E), all four lambs and three weaners were inoculated (i/v) with 2ml of a mixture of virus 1 and virus 2 (1:1 v/v), and two weaners were inoculated with 2ml (i/v) of virus 1 and virus 2 each.

The animals were monitored for discomfort and clinical signs twice daily, and temperatures were recorded every day. Normal pig and sheep temperatures were regarded as 38.7 - 39.8^{0}C and 38.3 - 39.9^{0}C, respectively (Robertshaw, 2004). Resting temperatures of 38^{0}C were also recorded as normal since the animals did not show signs of discomfort. Scoring of clinical signs was performed per species (Table 1 and.2). Sera, blood, and oral, nasal and rectal swabs were collected at days post infection (DPI) 0 to 7, then at 14, 21, 28 and 60 DPI if the animal was still alive.

Two suckling piglets and one weaner were randomly selected, starting with the infected ones, and euthanised in groups 1 and 2 by intracardiac injection with a barbiturate overdose (Eutha-naze, Bayer Health Care, Animal health, South Africa) every two days from DPI 2 respectively, while the remaining pigs were euthanised on DPI 60. Lactating sows were euthanised when there were no

suckling piglets left, by stunning with a captive bolt pistol followed by severing of the carotid artery to ensure death. Pregnant sows were euthanised following termination of pregnancy or farrowing using the same method utilised for lactating sows. Their newborn piglets were given an overdose of a barbiturate intracardially after birth. The weaners in group 3 were euthanised on DPI 30. Euthanasia of the lambs in all groups was indicated when they were too ill to feed and interact with their surroundings normally, as per experimental end-point scores approved by the animal ethics committee, or on DPI 30, using the same method applied in suckling piglets and weaners.

2.2.3 Laboratory Tests

a). Serology

The competitive ELISA kit for the detection of anti-Rift Valley fever (RVF) antibodies in ruminant serum or plasma (ID Screen® Rift Valley Fever Competition Multi-species, Louis Pasteur, France) was used for RVFV antibody detection. The assay is a multispecies test applicable for use on ruminants, horses, dogs and other species. Porcine and ovine sera from all experiments (n = 495) were tested according to the manufacturer's instructions. Sera with Sample/Negative percentage (S/N%) less than or equal to 40 were regarded as positive, those between 40 and 50 were deemed doubtful, and samples above 50 were considered negative for RVFV antibodies. For the purpose of this study, all doubtful results were regarded as positive.

b). Virus Isolation

Virus isolation was performed on 1/10 suspensions of pooled organs (n = 85) and terminal blood (n = 64) samples of all pregnant sows and their offspring, and pooled organs (n = 47) and terminal bleeds (n = 33) of control lambs and ewes, lactating sows, suckling piglets and weaners (n = 47), from groups 1, 2 and 3, using standard methods (OIE, 2019).

c). Viral RNA extraction, real-time and conventional RT-PCR

Total RNA from blood, oral, nasal and rectal swab pools, and homogenated organ samples, was extracted at the ARC-OVR Biotechnology PCR Laboratory using the magnetic-bead capture MagMAX-96 total RNA Isolation kit (MagNA Pure LC Instrument, Roche, South Africa). A published real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assay was used to test blood (n = 140), pooled organs (n = 107) and oronasorectal swabs (n = 83) from infected pregnant sows and their offspring. Blood (n = 168), pooled organs (n = 59) and oronasorectal swabs (n = 193) of pigs and control lambs from experiments involving infection of suckling piglets and weaners, and uninfected ewes and lactating sows, were also tested (Drosten et al., 2002).

For conventional RT-PCR, nucleic acid extraction was performed using E.Z.N.A Viral RNA kit (Omegabio-tek, U.S.A) and TRIzol reagent (Invitrogen & Thermofisher, U.S.A) according to the respective manufacturer's instructions. The Kifaro et al. (2014) RT-PCR method was used to test tissue cultured (TC) organ pool material from the three infection groups, constituting pregnant sows (n = 9), newborn piglets (n = 76), weaners (n = 9), ewes (n = 2) and lambs (n = 4), using a OneStep RT-PCR kit (QIAGEN, USA). The amplicons were mixed with 2 µL loading dye (Promega, USA), loaded on a 1% agarose gel (SeaKem, USA) containing 2 µL ethidium bromide (Promega, USA), together with a molecular weight marker of 1.5kb (Promega, USA), electrophoresed at 120 volts for 20 - 30 minutes, and then visualised under UV light for identification of positive samples (551 bp amplicons).

d). Viral RNA sequencing and phylogenetic analysis

The correct size amplicons generated from the conventional RT-PCR (n =17) were purified directly from the tube using the Roche High Pure PCR Product Purification Kit (Roche Diagnostics, South Africa). Bidirectional Sanger sequencing was performed on clean products with each of the PCR primers in separate reactions using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystem, South Africa) and submitted to the core Sanger sequencing facility of the University of Pretoria (South Africa).

Sequence chromatograms were edited and uploaded in the basic local alignment search tool (BLAST) for identification and selection with closely-related nucleotide sequences available in the Genbank database (www.ncbi.nlm.nih.gov). Sequences were aligned using ClustalW in MEGAX (Kumar et al., 2018) and end-unaligned regions were trimmed prior to generating summary statistics in MEGAX. The final dataset (353 nucleotides in length) was used to infer a neighbor-joining tree (Saitou & Nei, 1987), using the best-fit model identified under the Bayesian Information Criterion, with 10,000 bootstrap replicates performed to evaluate nodal support (Felsenstein, 1985).

e). Pathology

Post-mortems were conducted in the post mortem hall of the BSL3 animal facility following death of all experimental animals. Organ, blood and sera samples were collected for demonstration of anti-RVFV antibodies, RVFV RNA detection, virus isolation, histopathological examination, anti-RVFV immunohistochemistry and electron microscopic imaging.

For histopathology, liver, spleen and kidney samples collected in 10% neutral buffered formalin were embedded in paraffin wax using the standard protocol of the histopathology laboratory at the

University of Pretoria, Faculty of Veterinary Science. Histopathology lesions were scored according to species (Tables 3 and 4).

Immunohistochemistry for RVFV antigens was conducted on duplicate tissue sections using a polyclonal mouse ascitic fluid and an avidin-biotinylated peroxidase complex (ABC) immunodetection technique as previously described (Odendaal et al., 2014). Briefly, the standard immunoperoxidase method included routine deparaffination with two changes of xylene, rehydration through graded alcohol baths to distilled water, and incubation with 3% hydrogen peroxide for 15 minutes. This was followed by heat-induced epitope retrieval in citrate buffer (pH 6.0), followed by incubation with the anti-RVFV primary antibody (1:500) for 1 hour. Sections were sequentially incubated with the rabbit-anti-mouse secondary antibody (F0232, DakoCytomation, Glostrup, Denmark) followed by detection with a standard avidin-biotin peroxidase system, Vectastain® Elite® ABC-HRP Kit (PK-6100, Vector Laboratories, Inc., Burlingame, CA, USA), NovaRED peroxidase substrate (SK-4800, Vector Laboratories, Inc.) and haematoxylin counterstain. Slides were examined for positive labelling, typified as fine diffuse to coarse granular cytoplasmic brownish labelling using a light microscope. All microscopic images were captured with a DP25 camera (Olympus, Tokyo, Japan) on a BX46 light microscope (Olympus) using CellSens Standard Version 1.12 (Olympus).

f). Electron microscopy

The livers of aborted foetuses 1, 2, 5 and 10 from pregnant sow 5 in stable A were homogenised in PBS (1/10), centrifuged at 3000 rpm for 15 minutes, their supernatants collected and centrifuged at 13000 rpm for 45 minutes, with the resulting supernatant discarded and a drop of double distilled water poured on the sediment, followed by a drop of phosphotungstic acid. Standard negative staining transmission electron microscopy (TEM) techniques for identification of RVF virions were performed at the electron microscopy unit at the University of Pretoria with a few modifications (Ohi et al., 2004).

2.2.4 Statistical analysis

Differences in values of key experimental parameters such as proportion seropositive, temperatures, clinical and histopathological scores and Ct – values between groups were compared statistically. Paired data were analysed using paired t-test and independent data sets were evaluated using unpaired t-test and Mann Whitney U Test (https://www.graphpad.com/quickcalcs/ttest1.cfm; Statistics kingdom, 2017). Differences in proportions and binary data sets were evaluated using Comparison of proportions calculator and Fishers exact test, respectively (MedCalc Software Ltd. Calculators, Version 20.110).

2.3 Results

2.3.1 Clinical signs

a). Pregnant sows and offspring

There was one abortion 10 days before the expected farrowing date in a group 1 sow infected with M66/09 virus variant (Fig. 1). The remaining sows in groups 1 and those in group 2 infected with M21/10 virus variant, farrowed 1 to 7 days before the expected date and did not display overt clinical signs or discomfort. Rectal temperatures in both groups remained within the normal range of 38^{o}C – 39.8^{o}C ($P > 0.05$) (Fig. 2). Temperatures of newborn piglets (P) were not recorded in both groups, but stillborns, neonatal deaths, small and weak piglets, and those with congenital abnormalities were observed (Fig. 1). Median clinical scores for groups 1 and 2 were 0.52 and 1.7 respectively, and their distribution did not differ significantly (Mann–Whitney $U = 6$, $group_1 \neq group_2$, $P = 0.4$ two tailed), and neither did they differ significantly with those of their respective control lambs ($P > 0.05$).

b). Suckling piglets and weaners

No overt clinical signs were observed among infected suckling piglets and weaners in groups 1 and 2, and those infected with a mixture of M66/09 and M21/10 virus variants in group 3. Their temperatures remained within the normal range, except for slight pyrexia observed in a few animals, mainly on 1 DPI, including one control suckling piglet in group 1 (Table 2; Fig. 2). Mean temperature differences within and between groups were not significant ($P > 0.05$). Suckling piglets and weaners in groups 1 and 2 had median clinical scores of 1 and 0 respectively, which did not vary significantly (Whitney $U = 160$, $group_1 = group_2$, $P = 0.17$ two tailed). Group 2 pigs and their respective control lambs had median clinical scores of 0 and 3 respectively, whose distribution varied significantly (Mann–Whitney $U = 32$, $group_1 \neq group_2$, $P = 0.016$ two tailed).

c). Control lambs, ewes and lactating sows

Infection control lambs in groups 1 (n = 2) and group 3 (n = 4) showed pyrexia for the first 5 DPI and on 6 DPI respectively. Group 2 lambs showed severe clinical signs without temperature rises and were euthanised on 3 DPI (Fig. 2; Table 2). Uninfected lactating sow and ewe (n = 1 each) in group 1 had fluctuating temperature rises above 40^{0}C between 2 and 7 DPI, while the ewes in group 3 (n = 4) showed the same results between 1 and 11 DPI.

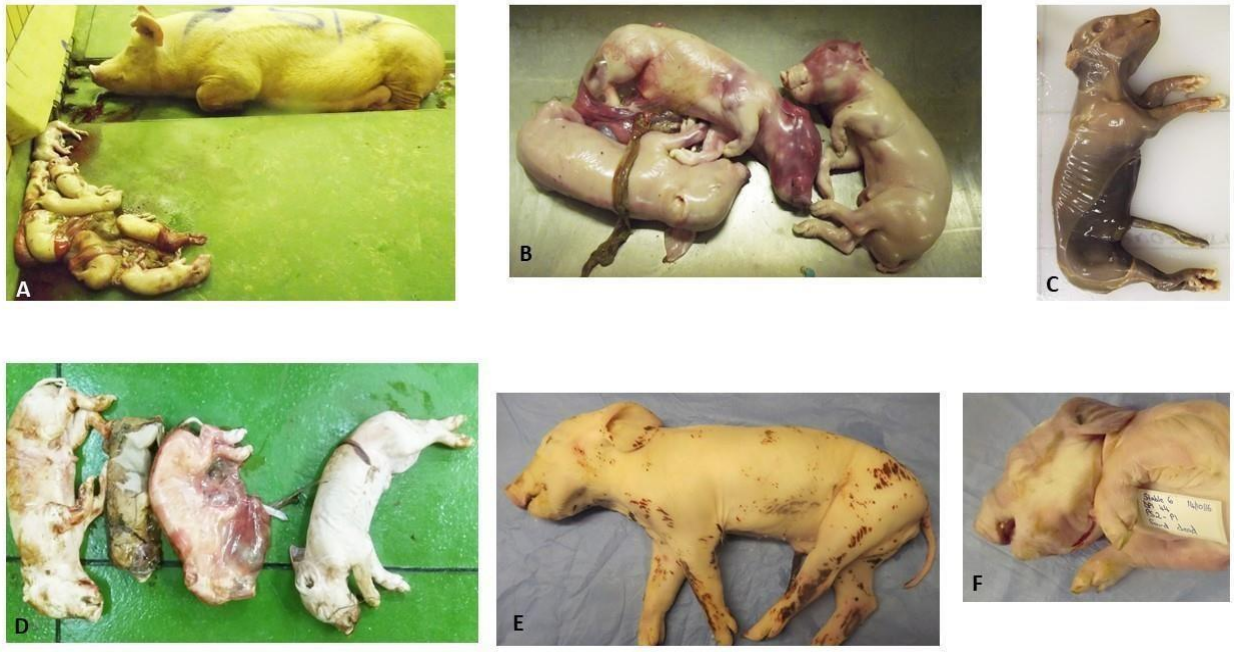

Figure 2.1 A: PS5A and aborted foetuses in group 1; **B**: Aborted foetuses presenting with congested skin over the head area and arthrogryposis; **C**: Mummified foetus expelled by PS5A in group 1; **D**: Dead piglets (stillborn) born from PS4C in group 2; **E:** Group 2's PS2-P3C with splayed left hind limb and decubitus ulcer on the medial aspect of the right hind leg; and **F**: Group 2's P2-P1C with brachycephalus and arthrogryposis

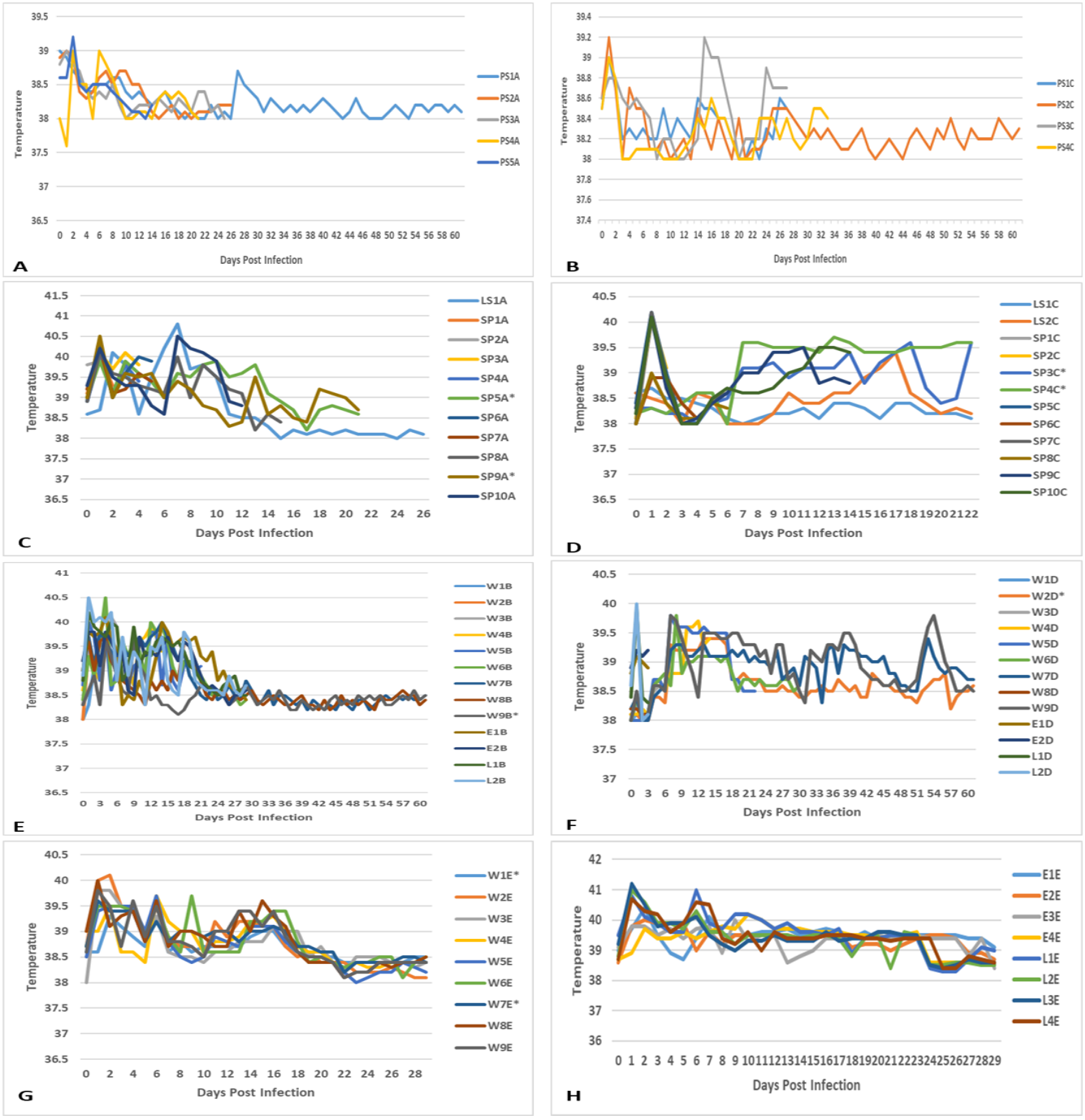

Figure 2.2 Temperatures (Degrees Celsius) of: **A**: pregnant sows (PS) infected with RVFV 1 (M66/09 variant) in group 1 from 0-61 days post infection (DPI); **B**: pregnant sows (PS) infected with RVFV 2 (M21/10 variant) in group 2 from 0-61 days post infection (DPI); **C**: suckling piglets (SP) infected with RVFV – M66/09 variant and lactating sow (LS) in group 1; **D**: suckling piglets (SP) infected with RVFV – M21/10 variant and lactating sows in group 2; **E**: weaners (W) and control lambs (L) infected with RVFV – M66/09 variant, and uninfected ewes (E) in group 1; **F**: weaners (W) and control lambs (L) infected with RVFV – M21/10 variant, and uninfected ewes (E) in group 2; **G**: uninfected control weaners (W; W1 and W7), those infected with RVFV M66/09 variant (W2 and W5) and M21/10 variant (W4 and W6) only, and those infected with a mixture of the two viruses (W3, W8 and W9) in group 3; **H**: control lambs (L) infected with RVFV mixture (M66/09 and M21/10 variants) and uninfected ewes (E) in group 3

Table 2.1 Clinical score descriptions of infected pigs and control lambs

Clinical Score	Description
0	No clinical signs
1	Pyrexia
2	Small size and weakness (porcine) Weakness (ovine)
3	Alive with splay legs, arthrogryposes, umbilical hernia and other abnormalities (porcine neonates) Disinclination to move, anorexia and weakness (suckling ovines and porcines, and weaners)
4	Neonatal mortality; stillborn; mummies, fresh and macerated foetuses with or without abnormalities
5	Abortion (sows)

Table 2.2 Comparison of clinical scores of pregnant sows and their offspring, suckling piglets and weaners, and weaners in groups 1 (M66/09 variant), 2 (M21/10 variant) and 3 (mixture of M66/09 and M21/10 variants) respectively, and their corresponding control lambs. Negative control animals were excluded from scoring

M66/09 virus variant inoculated animals	No. of animals (n)	Average score	Observations
PS1A	**0**	**0**	**N/A**
PS2A and piglets	**17**	**0.375**	**Weakness and lameness (n =1); umbilical hernia (n=1); normal (n = 15)**
PS3A and piglets	**15**	**0.67**	**Small and weak (n=1); dead (n=2); normal (n = 12)**
PS4A and piglets	**8**	**0**	**Sow and all piglets healthy**
PS5A and foetuses	**21**	**4.05**	**Abortion (n=1); dead foetuses (n = 14, of which two had arthrogryposis); mummified foetuses (n = 2); macerated foetuses (n = 4)**
Average score	**1.273**		
SP1A, SP2A, SP3A, SP4A, SP6A, SP7A, SP8A and SP10A	**8**	**0.75**	**Pyrexia (n = 6); normal (n = 2)**
Average score	**0.75**		
W1B, W2B, W3B, W4B, W5B, W6B, W7B, and W8B	**8**	**0.5**	**Pyrexia (n = 4); normal (n = 4)**
Average score	**0.5**		
L1B	**N/A**	**1**	**Pyrexia**
L2B	**N/A**	**1**	**Pyrexia**
Average score	1		

M21/10 virus variant inoculated animals	No. of animals (n)	Average score	Observations
PS1C and piglets	18	1,222	Weak (n = 1); dead (n = 5); normal (n = 12)
PS2C and piglets	10	1.7	Dead (n = 2; all with arthrogryposis and 1 with brachycephalus); alive with splay legs (n = 3); normal (n = 5)
PS3C and piglets	12	0.167	Weak (n = 1); normal (n = 11)
PS4C, stillborns and piglets	9	1.78	Stillborn (n = 4); normal (n = 5)
Average score	1.22		
SP1C, SP2C, SP5C, SP6C, SP7C, SP8C, SP9C, and SP10C	8	0.75	Pyrexia (n = 6); normal (n = 2)
Average score	0.75		
W1D, W3D, W4D, W5D, W6D, W7D, W8D, and W9D	8	0	All normal
Average score	0		
L1D	N/A	3	Disinclination to move, anorexia, weak
L1D	N/A	3	Disinclination to move, anorexia, weak
Average score	3		
M66/09 and M21/10 virus variant mix inoculated animals	No. of animals (n)	Average score	Observations
W3E	N/A	1	Pyrexia
W8E	N/A	0	None
W9E	N/A	0	None

M66/09 and M21/10 virus variant mix inoculated animals	No. of animals (n)	Average score	Observations
Average score	**0.33**		
L1E	**N/A**	**1**	**Pyrexia**
L2E	**N/A**	**1**	**Pyrexia**
L3E	**N/A**	**1**	**Pyrexia**
L4E	**N/A**	**1**	**Pyrexia**
Average score	1		

N/A: Not applicable

2.3.2 Pathology

2.3.2.1 Macroscopic observations:

a). Pregnant sows and resultant offspring

Pregnant sows did not show gross macroscopic lesions or abnormalities, but a few external observations were made from piglets that were born ill and weak, and those that died shortly after birth, either from natural causes or euthanasia. The affected animals had smaller carcasses compared to litter mates (group 1: n = 2; group 2: n = 5) and poor condition scores of approximately 1.5/5 to 2/5 (group 2: n = 6). Arthrogryposis (group 2: n = 2), splay legs with associated decubitus ulcers (group 2: n = 3); and umbilical hernia were observed (group 1: n = 1) (Fig.2). Internal lesions of varying severity and distribution patterns were seen and mostly involved the liver, kidney and spleen (Fig. 3). Congestion and a few haemorrhages were the main observations associated with the gastrointestinal tract (GIT). Almost all major organ systems of the aborted foetuses (group 1) and stillborns (group 2) exhibited lesions (Fig. 3).

The lesions observed in the foetuses and stillborn pigs are described below:
Central nervous system: cerebral and cerebellar hypoplasia (group 1: n = 1); brains with jelly-like and semi-liquefied cerebrum and cerebellum (group 1: n = 10), and those with no clear delineation between the grey and white matter (group 2: n = 2); congestion of the brain, meninges and blood vessels (group 1: n = 5; and group 2: n = 4); and a pink, soft and friable spinal cord (group 1: n = 1) (Fig. 3).Other than cerebral and cerebellar hypoplasia, these observations may be attributable to post-mortum changes.

Circulatory system: pallor of the myocardium (group 1: n = 5; group 2: n = 3), haemorrhages (group 1: n = 8) and congestion (group 1: n = 1); blood tinged (group 1: n = 4; and group 2: n = 1) and straw coloured (group 1: n = 3; group 2: n = 1) hydrothorax were present also in a neonate (group 1: n =

1) (Fig. 3); clear (group 1: n = 2) and blood tinged (group 2: n = 1) ascitic fluid in the abdominal cavities and haemoperitoneum (group 1: n = 1) (Fig. 3). Many of these observations may be attributable to post-mortum changes, but those involving haemorrhage or transudates in body cavities may also be due to RVFV infection.

Respiratory system: lung lesions included oedema (group 1: n = 2; group 2: n = 1), congestion and pulmonary consolidation (hepatisation) coupled with oedema in some (group 1: n = 5; group 2: n=1), and a combination of oedema, congestion and haemorrhage (group 1: n = 4; group 2: n = 1) (Fig. 3). The changes are highly likely to be RVFV infection related.

Digestive and hepatobiliary systems: liver lesions varied in degrees of severity and different distribution patterns. Regular postmortem changes included friability, dark red discolouration and congestion. These findings are common in ovine foetuses (Odendaal et al., 2020b) and may be post-mortem changes in porcines as well. Lesions that may be attributable to RVFV infection include haemorrhages, hepatic necrosis (1-2mm) (group 1: n = 4), and pallor or diffuse yellow discoloration (group 2: n = 2) (Fig. 3). In the GIT, congestion of the mucosa was the only post-mortem finding. (group 1: n = 8; group 2, n = 3) (Fig. 3).

Urogenital system: Kidney lesions in the aborted foetuses (group 1) included enlargement, friability, congestion, haemorrhages and infarcts (1 - 5mm). Capsules were hard to peel over the necrotic areas, leaving rough surfaces. The kidneys of stillborn pigs were pale, pulpy and one was severely congested with an infarct (group 2: n = 4). The testicles (group 1: n = 2) were very small and muscles (group 1: n = 2) showed generalised congestion. The congestion and friability may be post–mortem changes.

Immune system: Spleens were pale-pink and friable (group 1: n = 8), and haemorrhagic (group 1: n = 1); and others displayed haemorrhages and redness, pallor and pulpiness, and haemorrhages and infarcts (group 2, n = 3).Similar to the urogenital system, the colour changes and friability may be post-mortem changes.

b). Suckling piglets and weaners

Lesions were mostly seen on the liver, spleen and kidneys. These organs presented with congestion, haemorrhage and necrosis of varying severity and distribution (Fig. 3).

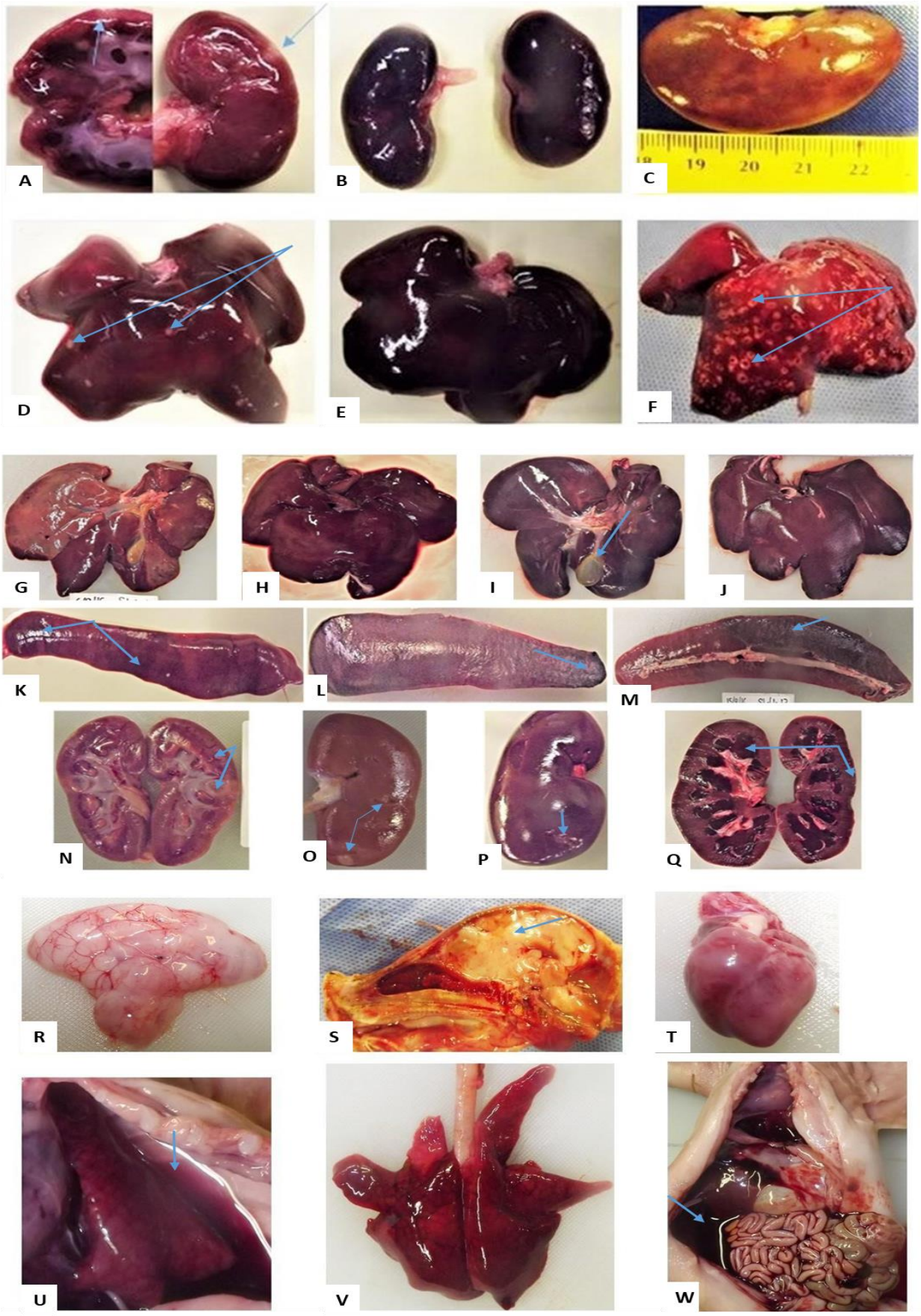

Figure 2.3 Suffixes A and B (group 1), C and D (group 2), and E (group 3), at the end of each animal ID denotes their stable numbers, and lesions are pointed with blue arrows: **A**: Infarct in cortex and medulla of PS5A-AF1 kidney; **B**: Congestion and haemorrhage of PS5A-AF3's kidneys; **C**: Soft, enlarged kidney of PS2C-P9 with diffuse pale-yellowish areas; **D**: Infarcts on the liver of PS5A-AF2; **E**: Congestion and haemorrhage of PS5A-AF3's liver; **F**: Multifocal necrotic foci on PS5A-AF10's liver giving it nutmeg appearance; **G**: Congested liver of SP5C on DPI 4; **H**: Congested liver of SP6A at DPI 6; **I**: Congested liver of W4D on DPI 15, with oedematous gall bladder; **J**: Congested liver of W3B on DPI 6; **K**: Enlarged spleen of SP5C with haemorrhages; **L**: Enlarged spleen of W4D on DPI 15 with haemorrhages; **M**: Spleen of W3B on DPI 6 with haemorrhages; **N**: Kidney of SP7A on DPI 6 with infarcts; **O**: Kidney of SP8C on DPI 15 with infarcts; **P**: Kidney of W4D in on DPI15, with infarcts and a rough cortical surface remaining following peeling of the capsule; **Q**: Severe congestion and tubular degeneration of a kidney of W3B on DPI6, **R**: Soft and jelly-like brain of PS5A-AF1; **S**: Brain of PS5A-AF1 with unclear delineation between white and grey matter; **T**: Soft and pale heart of PS5A-AF2; **U**: PS5A-AF3's flat congested lung and blood tinged hydrothorax; **V**: Oedema, congestion and haemorrhage of PS5A-AF1's lung; **W**: Blood tinged ascites in PS5A-AF2 and congested GIT

2.3.2.2 Histopathology and Immunohistochemistry:

Histopathological lesions were assigned scores as described in Table 3, and the scores were compared among the different treatment groups (Table 4). Histopathological examinations were performed on liver (n = 153), kidney (n = 150) and spleen (n = 150) samples, and only a limited number of livers (n = 76), spleens (n = 21) and kidneys (n = 11) were subjected to IHC testing. The IHC signals were faint, most probably due to the low RVF antigen concentrations in the analysed tissues. Since uninfected in-contact pigs analysed were proven to be horizontally infected in this study, there were no negative pig tissue controls for comparison, and the observed faint IHC signals could be false positives.

a). Pregnant sows and resultant offspring

The liver of one sow per group was analysed and only the sow in group 2 showed hepatocyte swelling. Aborted piglets or those born from infected sows in groups 1 and 2 displayed lesions as described in Table 4 and Figure 4. Both groups had a median histopathological score of 1 and the distributions did not differ significantly (Mann-Whitney U = 948, $group_1 \neq group_2$, P = 0.269 two-tailed). However, significant median distribution differences described by Mann-Whitney U = 71.5, $group_1 \neq group_2$, P = 0.02 two-tailed, and Mann-Whitney U = 2, $group_1 \neq group_2$, P = 0.00 two-tailed, were observed between group 1 porcines (median = 1) and control lambs (median = 2), and group 2 porcines (median = 1) and control lambs (median = 0) respectively. A few livers (n = 6) tested positive on IHC (Table 4; Fig. 4). Development of hepatocyte glycogen storage vacuoles in hepatocytes is also normal in fed pigs (Cheon et al., 2005).

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b).Suckling piglets and weaners

Lesions observed in suckling piglets and weaners in all 3 groups are described in Table 4. The median histopathological scores for groups 1 and 2 were both 1 and their distributions were not

significantly different (Mann-Whitney U = 152, $group_1 \neq group_2$, P = 0.103 two-tailed). Significant differences in the median score distributions of group 1 pigs (median = 1) and their control lambs (median = 2) (Mann-Whitney U = 28.5, $group_1 \neq group_2$, P = 0.003 two-tailed), and group 2 pigs (median = 1) and their control lambs (median = 0) (Mann-Whitney U = 2, $group_1 \neq group_2$, P = 0.01 two-tailed), were nonetheless observed. Among the samples selected for IHC testing, suckling piglets (group 1: n = 3 and group 2: n = 2), and weaners (group 1: n = 2) showed positive staining for RVFV antigens in the livers only (Fig. 4). A number of livers from both groups of pigs presented with tiny scattered positive staining nonetheless. Fed pigs can normally develop hepatocyte glycogen storage vacuoles following feeding (Cheon et al., 2005).

Table 2.3 Histopathological score description. Adopted and modified from Faburay et al. (2016)

Histopathology Score	Description
0	No lesions attributable to Rift Valley Fever virus
1	i. Multifocal, mid-zonal to central foci of lymphohistiocytic (lymphocytes and macrophages) inflammation with or without presence of few plasma cells and single hepatocyte necrosis (ovines) ii. Hepatocyte swelling with or without presence of lymphohistiocytic (lymphocytes and macrophages) inflammation, few plasma cells and single hepatocyte necrosis (ovines and porcines)
2	Multifocal, 1–2 mm areas of mid-zonal to central lymphohistiocytic inflammation with central necrosis shifting inflammation to predominantly neutrophils. Less than 5% of examined parenchyma involved
3	Multifocal, 1–2 mm areas of mid-zonal to central lymphohistiocytic inflammation with central necrosis shifting inflammation to predominantly neutrophils. Approximately 15% of examined parenchyma involved, and scattered hepatocyte apoptosis present
4	Greater than 15% of the parenchyma is necrotic and severe multifocal haemorrhage is also present

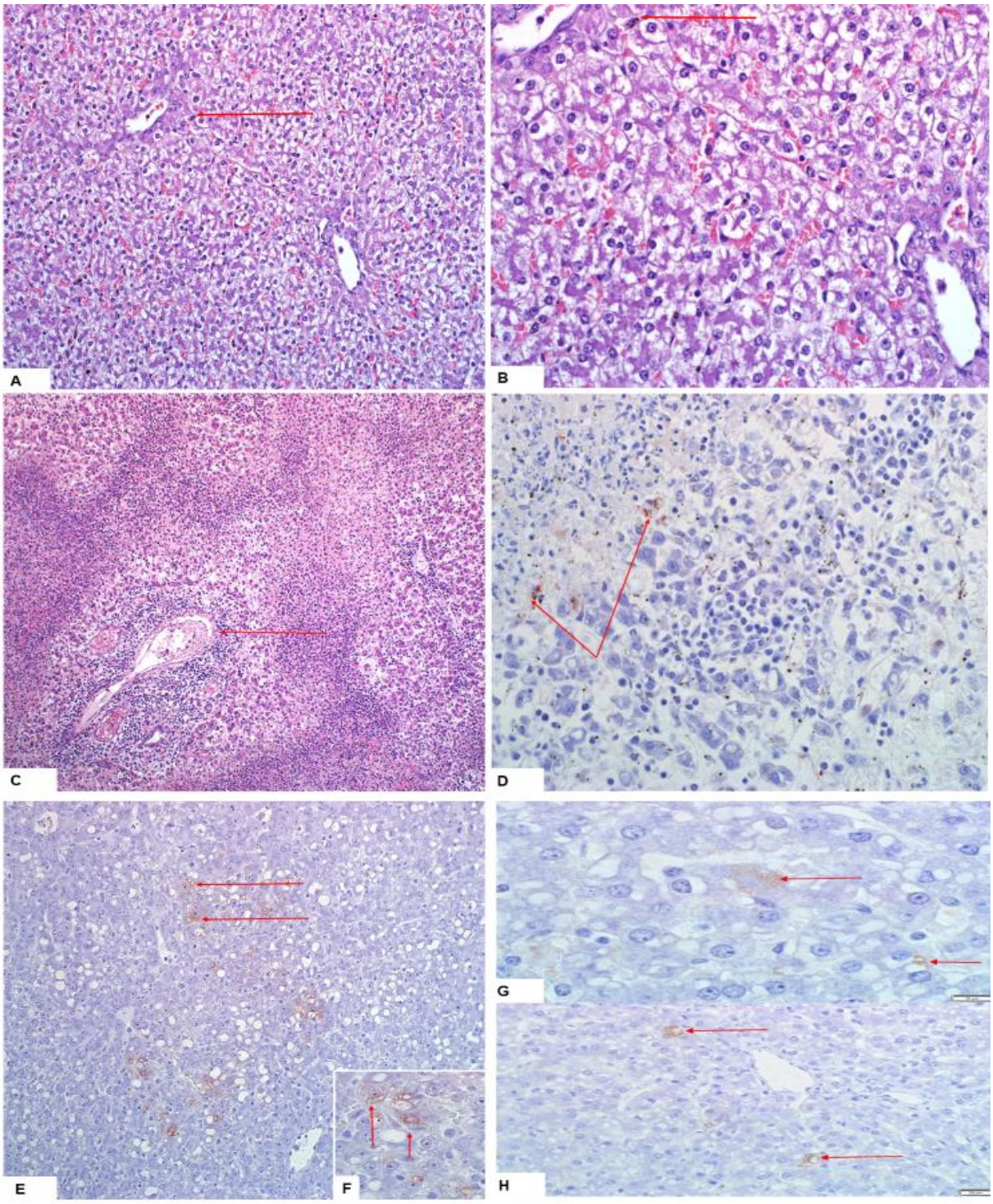

Figure 2.4 Lesions and RVFV antigen are indicated with red arrows: **A** and **B**: hepatocyte swelling with clarification of the cytoplasm of a neonate piglet born from a pregnant sow infected with M21/10 virus variant in stable C (H&E staining, 20X and 40X magnification); **C** and **D**: necrotic hepatocytes and infiltration of inflammatory cells around the hepatic portal vein of a PS5 foetus, and faint RVFV antigen foci on IHC staining of the same foetus from the M66/09 virus variant infected sow; **E** and **F**: positive antigen staining in liver of a neonate from a PS in group 1 (20X magnification and close-up view respectively); and **G** and **H**: Liver of SP2A showing RVFV 1 antigens in the hepatocytes (100X and 40X magnification respectively)

Table 2.4 Comparison of histopathological scores of livers from animals infected with virus or born from sows infected with virus in groups 1 (M66/09 variant), 2 (M21/10 variant), and group 3 (mixture of M66/09 and M21/10 variants). IHC scores means at least one liver in the group was positive. Lesions were either mild, moderate or severe

Animal I.D	No. of animals (n)	DPI	Average H-Score	Observations	IHC	H – Other organs
Group 1: Virus variant M66/09						
PS	1	61	1	Hepatocyte swelling (glycogen) and steatosis	NT	-
PS-AF	11	14	1	Hepatocyte vacuolation, swelling (glycogen) and steatosis	+	+ k: Congestion (n =4)
P	34	23 - 32	0.85	Hepatocyte vacuolation, swelling (glycogen) and steatosis; bile stasis; congestion; and wide spread single cell necrosis	+	+ k: Congestion (n = 3) + s: Congestion (n = 2)
SP	8	2 - 15	1	Hepatocyte swelling (glycogen) and steatosis	+	+ s: Congestion (n = 1) White pulp expansion (n = 1)
W	7	2 - 61	1	Hepatocyte swelling (glycogen) with or without leucostasis (polymorphonuclear and mononuclear)	+	+ s: White pulp expansion and congestion (n = 8) + k: Acute tubular injury; patchy PTE cell degeneration with pyknotic nuclei and detachment of cells from the tubular basement membrane (n = 3). Marked infiltrate of lymphoplasmacytic cells in the medullary interstitium (n = 1)

Animal I.D	No. of animals (n)	DPI	Average H-Score	Observations	IHC	H – Other organs
Group 1: Virus variant M66/09						
L	2	29	0	No lesions attributable to RVFV infection in ovines	-	-
Group 2: Virus variant M21/10						
PS	1	27	1	Hepatocyte swelling (glycogen) and steatosis	NT	**-**
SB	2	32	1	Hepatocyte vacuolation and swelling (glycogen).	NT	+ k: Congestion
P	41	22 - 44	0.95	Hepatocyte vacuolation, swelling (glycogen) and steatosis; bile stasis; congestion; and wide spread single cell necrosis	**-**	+ s: Congestions and haemosiderosis (n = 1) Congestion (n = 4) + k: Congestion and proximal tubular cell swelling (n = 2) Congestion (n = 2)

Animal I.D	No. of animals (n)	DPI	Average H-Score	Observations	IHC	H – Other organs
Group 2: Virus variant M21/10						
SP	6	2 - 22	1	Hepatocyte swelling (glycogen) and steatosis, with or without leucostasis (mononuclear); increased number of Kupffer cells in the sinusoids	**+**	**-**
W	7	2 - 62	1	Hepatocyte swelling (glycogen) and steatosis	**+**	+ s: White pulp expansion with or without congestion (n = 9) + k: Patchy PTE cell degeneration with pyknotic nuclei and detachment of cells from the tubular basement membrane (n = 4)

Animal I.D	No. of animals (n)	DPI	Average H-Score	Observations	IHC	H – Other organs
Group 2: Virus variant M21/10						
L	2	3	2.50	Random foci of necrosis with marked infiltrate of Kupffer cells and very few neutrophils; severe necrosis involving more than 75% of the specimen with an inflammatory infiltrate of Kupffer cells and very few neutrophils, and typical RVF primary foci and nuclear inclusions	+	+ s: Marked infiltrate of neutrophils in the red pulp (n = 1) + k: Subtle injury and loss of nuclei and pyknosis in the glomeruli and mild tubular injury with a few pyknotic nuclei and scattered detachment of cells from the tubular basement membrane of a few proximal tubules. (n = 1)
Group 3: Virus mix (M66/09 and M21/10)						
W	3	29	1	Hepatocyte swelling (glycogen)	-	+ k: Tubular injury with or without proximal tubular epithelial (PTE) cell degeneration with pyknotic nuclei and detachment of cells from the tubular basement membrane, and infiltration of lymphoplasmacytic cells in the medullary interstitium (n = 3) + s: marked white pulp expansion, with mild to severe congestion (n = 3)

Animal I.D	No. of animals (n)	DPI	Average H-Score	Observations	IHC	H – Other organs
Group 3: Virus mix (M66/09 and M21/10)						
L	4	29	1.25	Hydropic degeneration of hepatocytes with randomly scattered lymphocytes and neutrophils	NT	+ s: Congestion (n = 2) + k: Mild interstitial nephritis (n = 2).

ID: Identity; H: Histopathology; IHC: Immunohistochemistry; DPI: Days post infection; PS: Pregnant sow; AF: Aborted foetus; P: Piglet (newborn); SP: Suckling piglet; W: Weaner; SB: Still born; L: Lamb; NT: Not tested; s: Spleen; k: Kidney; +: Positive; -: Negative

2.3.3 Serology

a). Pregnant sows and resultant offspring

All pregnant sows (n = 5) in group 1 (M66/09 virus variant) seroconverted from 14 DPI, and remained positive until their humane euthanasia on different DPI. Anti-RVFV antibodies were detected in the offspring (n = 25; 75. 76%) of three of the sows which farrowed (Fig. 5). In group 2 (M21/10 virus variant), half the sows (n = 2) and their offspring did not seroconvert, while the remaining sows (n = 2; 50%) and their piglets (n = 16; 43.2%) seroconverted from 4 DPI (Fig. 5). All group 1 control lambs (n = 2) seroconverted, but group 2 control lambs did not (n = 2). The proportion of seropositives between group 1 and group 2 sows and their offspring were significantly different (P = 0.0015) (Appendix 2 and 3).

b). Suckling piglets and weaners

In group 1, a mock infected sucking piglet (n = 1) and weaners (n = 3), and a negative control weaner (n = 1) seroconverted. For group 2, an infected suckling piglet (n = 1) and weaners (n = 6), and a mock infected weaner (n = 1) demonstrated antibody presence. Differences in the proportion of seropositive infected suckling piglets and weaners between the two groups were not significant (P = 0.27). Among the animals infected with a mixture of M66/09 and M21/10 virus variants in group 3, control lambs (n = 4) and a weaner (n = 1) demonstrated antibody presence (P = 1.00) (Appendix.4).

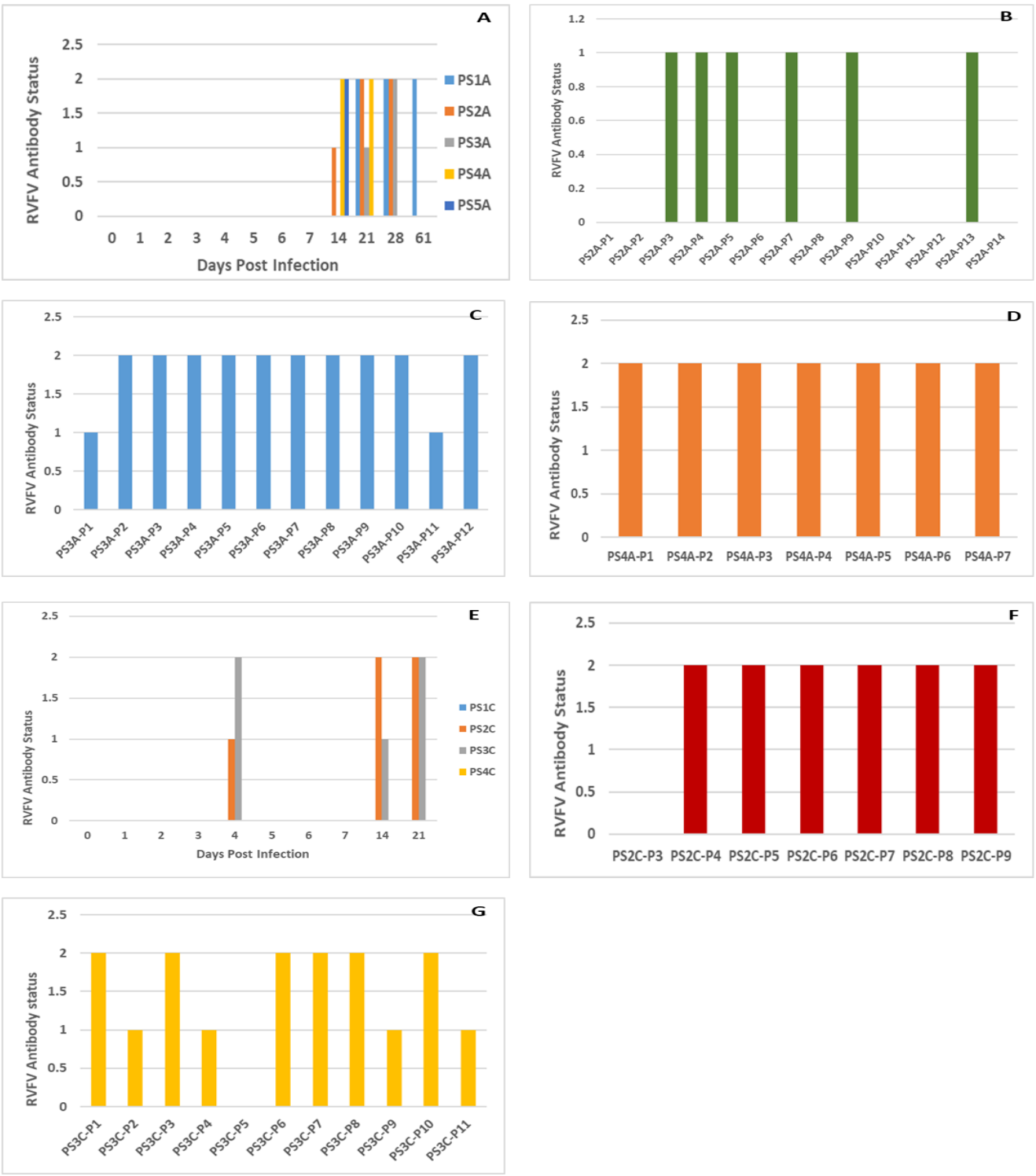

Figure 2.5 A: IDVet Blocking ELISA detected antibody statuses of: **A**: Pregnant sows (PS) infected with RVFV1 in stable A (S/N% mean: 16.40); **B**, **C** and **D**: Piglets born from PS2A (S/N% mean: 53.73), PS3A (S/N% mean: 32.17) and PS4A (S/N% mean: 27.57) in stable A on 23, 28 and 32 DPI respectively; **E**: PS infected with RVFV 2 in stable C (S/N% mean: 57); Piglets born from PS2C (S/N% mean: 33.85)and PS3C (S/N% mean: 42) in stable C on 44 and 22 DPI respectively. Antibody statuses of 0, 1 and 2 denote negative, suspect and positive, respectively.

2.3.4 Real time RT-PCR

a). Pregnant sows and resultant offspring

Rift valley fever virus RNA was detected in a few organ and oronasorectal swab pools, and blood samples from both group 1 and 2 (Appendix 2 and 3). No RVFV RNA was detected in the organ pool and blood samples of the infected pregnant sows in the two groups. However, in group 1, oronasorectal swab pools from two pregnant sows each tested positive on 3 and 4 DPI, and on 2 and 4 DPI, while an oronasorectal swab pool from a single pregnant sow in group 2 tested positive on 21 DPI. In group 1, organ pool samples from aborted foetuses (n = 2) and newborn piglets (n = 14) from sows (n = 4), and one blood sample collected 28 DPI from a newborn piglet, yielded positive results. Positive results in group 2 were obtained from organ pool samples of stillborn (n = 1) and newborn piglets (n = 19) from all infected sows (n = 4), and from blood collected on 27 DPI from a newborn piglet. Ct-values in groups 1 and 2 ranged from 18.97 – 39 (median: 35.8) and 23.97 – 38 (median: 34.15), respectively, and their median distributions were not significantly different (Mann-Whitney $U = 136$, $group_1 \neq group_2$, $P = 0.13$). Median Ct-value distributions of group 2 sows and litters and their control lambs were significantly different (Mann-Whitney $U = 0$, $group_1 \neq group_2$, $P = 0.02$).

b). Suckling piglets and weaners

Five organ pools tested positive in group 1, including suckling piglets (n = 3), weaner (n = 1), and positive control lamb (n = 1). The blood of one newborn piglet, and oronasorectal swab pools of a suckling piglet, weaners (n = 2) and a lamb tested positive. Group 2 recorded ten positive organ pool samples, including those from suckling piglets (n = 3), a weaner (n = 1), positive control lambs (n = 2), and uninfected lactating sows (n = 2) and ewes (n = 2). Blood of weaners (n = 2) and a control lamb (n = 1) tested positive. Oronasorectal swab pools of a suckling piglet (n = 1) and weaners (n = 4), and negative control weaner (n = 1) also tested positive in this group (Appendix2 and 3). Ct-values ranged from 15 – 35.22 (median: 34.47) and 26.47 – 33.98 (median: 32.51) in groups 1 and 2, respectively, and their distributions did not vary significantly (Mann-Whitney $U = 11$, $group_1 \neq group_2$, $P = 0.1$). Median Ct-value distributions of group 2 porcines (median = 32.51) and their control lambs (median = 16.26) were significantly variable (Mann-Whitney $U = 0$, $group_1 \neq group_2$, $P = 0.04$).

In group 3, organ pool samples of an infected weaner (n = 1) and an uninfected control weaner (n =1), and a blood sample from a control lamb (n = 1), tested positive (Appendix 4).

2.3.5 Virus isolation and conventional RT-PCR

a). Pregnant sows and their offspring

One to three passages were performed per sample. Atypical Vero cell morphology, which was probably CPE, was observed following inoculation with the experimental porcine samples when compared with the cell controls for approximately 50% of all the organ pools and blood tested (Fig.

6). Presence of RVFV RNA in TC supernatants was only determined for organ pool samples which consistently yielded CPE–like appearance on cell culture in subsequent blind passages. Blood samples only underwent a single passage in cell culture and the consistency of their effect on the cell monolayers was not verified. Conventional RT-PCR yielded 17/44 (38.6%) and 18/42(42.85%) positive results for group 1 and 2 organ pool samples respectively (P = 0.69).

b). Suckling piglets and Weaners

Similar to the results obtained from samples of the pregnant sows and their offspring, atypical vero cell morphology was observed following infection with organ pool samples from groups 1 to 3, comprising of ewes (n = 8), lambs (n = 8), lactating sows (n = 3), suckling piglets and weaners (n = 40), and their terminal bleeds (n = 30), in approximately 30% of the flasks. Of the samples tested by conventional RT-PCR, only one lamb from group 2 tested positive.

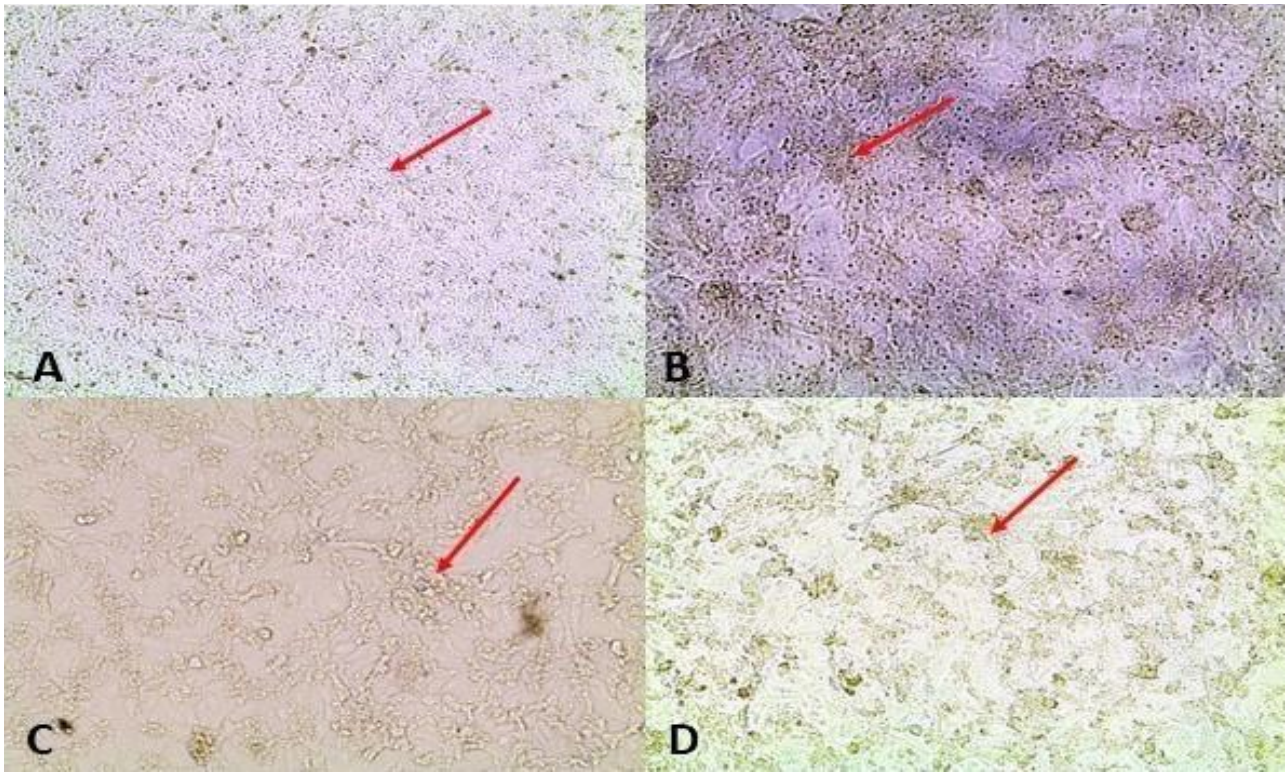

Figure 2.6 Red arrows point at normal cell monolayers and cytopathic effect: A). Three-day-old control Vero cells maintained with the same medium as the one used to isolate virus from pooled organ homogenate supernatants; B). Day 3 of neonate (group 1) organ pool passage 1 on Vero cells; C). Day 3 of neonate (group1) organ pool passage 1 on Vero cells; D). Day 3 of neonate (group 2) organ pool passage 1 on -Vero cells

2.3.6 Sequencing and Phylogenetic Analysis

a). RT-PCR

Among the samples tested by conventional PCR (n = 17), only 11 showed visible bands on agarose gel electrophoresis (Fig. 7), of which 9 were selected for purification and sequencing since they had acceptable nucleic acid concentrations. These were group 2 control lambs (n = 2), weaner (n = 1), a pregnant sow (n = 1) and her piglet (n = 1), group 1 piglets (n = 2), and two positive controls, *viz*. TC material from Onderstepoort Biological Products (OBP) and virus, M21/10.

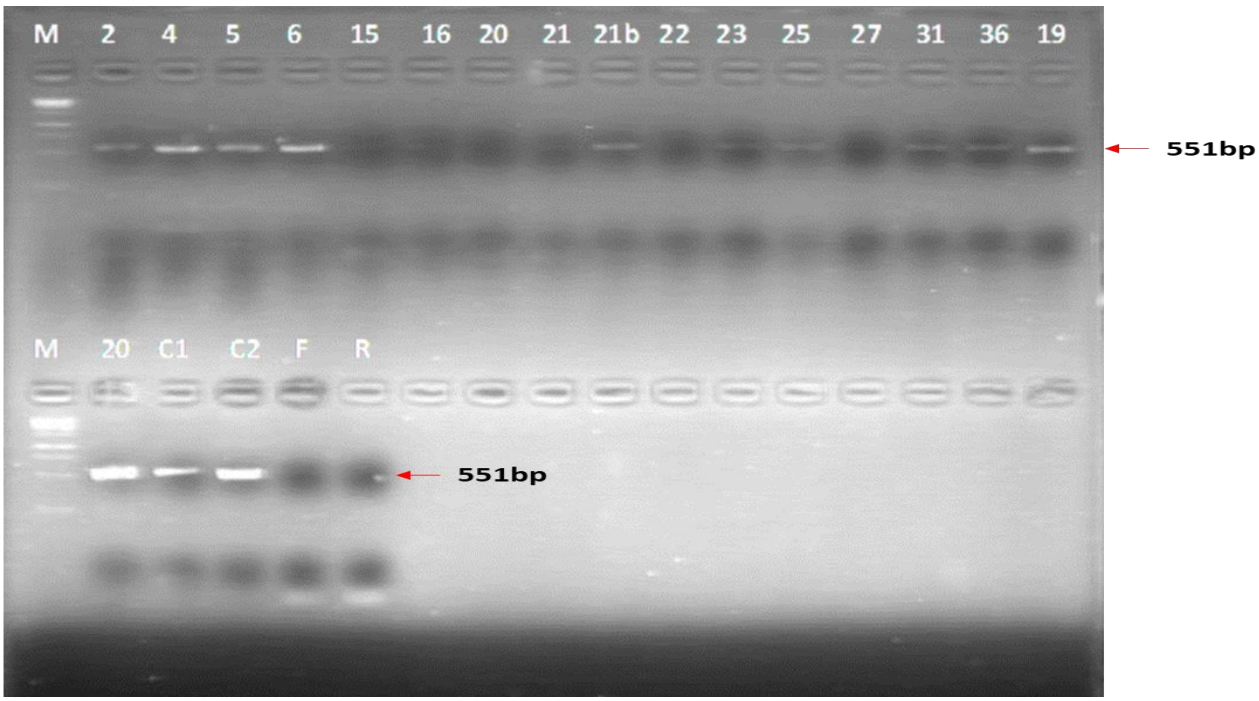

Figure 2.7 Results of the conventional RT-PCR run which informed the choice of samples to analyse for purity and sequencing. M = 1kb marker; 2 = PS2C Piglets (group 2); 4 = L1D (group 2); 5 = PS4C Piglets (group 2); 6 = PS2C (group 2); 15 = PS5A Foetuses (group 1); 16 = PS1A (group 1); 20 = PS2A (group 1); 21 = PS3A (group 1); 21b = W4D (group 2); 22 = PS1C (group 2); 23 = PS2A Piglets (group 1); 25 = PS3C (group 2); 27 = W1B (group 1); 31 = PS1C Piglets (group 2); 36 = PS3A Piglets (group 1); 19 = L1D (group 2); C1 = M21/10 virus Control; C2 = OBP TC positive control; F = Forward primer; R = Reverse primer

b). Sequencing and Phylogenetic Analysis

Sequences were successfully generated for six of the nine samples, and included a group 1 piglet infected with RVFV strain M66/09 variant (n = 1), group 2 infected animals (M21/10 variant), lamb (n = 1), weaner (n = 1) and pregnant sow (n = 1), and PCR positive controls M21/10 variant (n = 1) and OBP-TC virus (n = 1). Partial and full M-segment genome sequences (n = 37) were sourced from Genbank for confirmation of identity and comparison of genotypes, bringing the total number of taxa analysed to 44 (Fig. 8). End-unaligned sequences were removed, resulting in a final dataset of 353

nucleotides in length. Phylogenetic analysis revealed that sequences generated from groups 1and 2 animals clustered within lineages C and H with the M66/09 and M21/10 variants respectively, while the OBP tissue culture virus grouped within lineage K (Fig. 8). Percent identities between sequences generated in this study and selected reference sequences from the 2009 and 2010 RVF outbreak strains ranged from 96.88% to 100% and 91.45% to 100% at nucleotide and amino acid levels respectively.

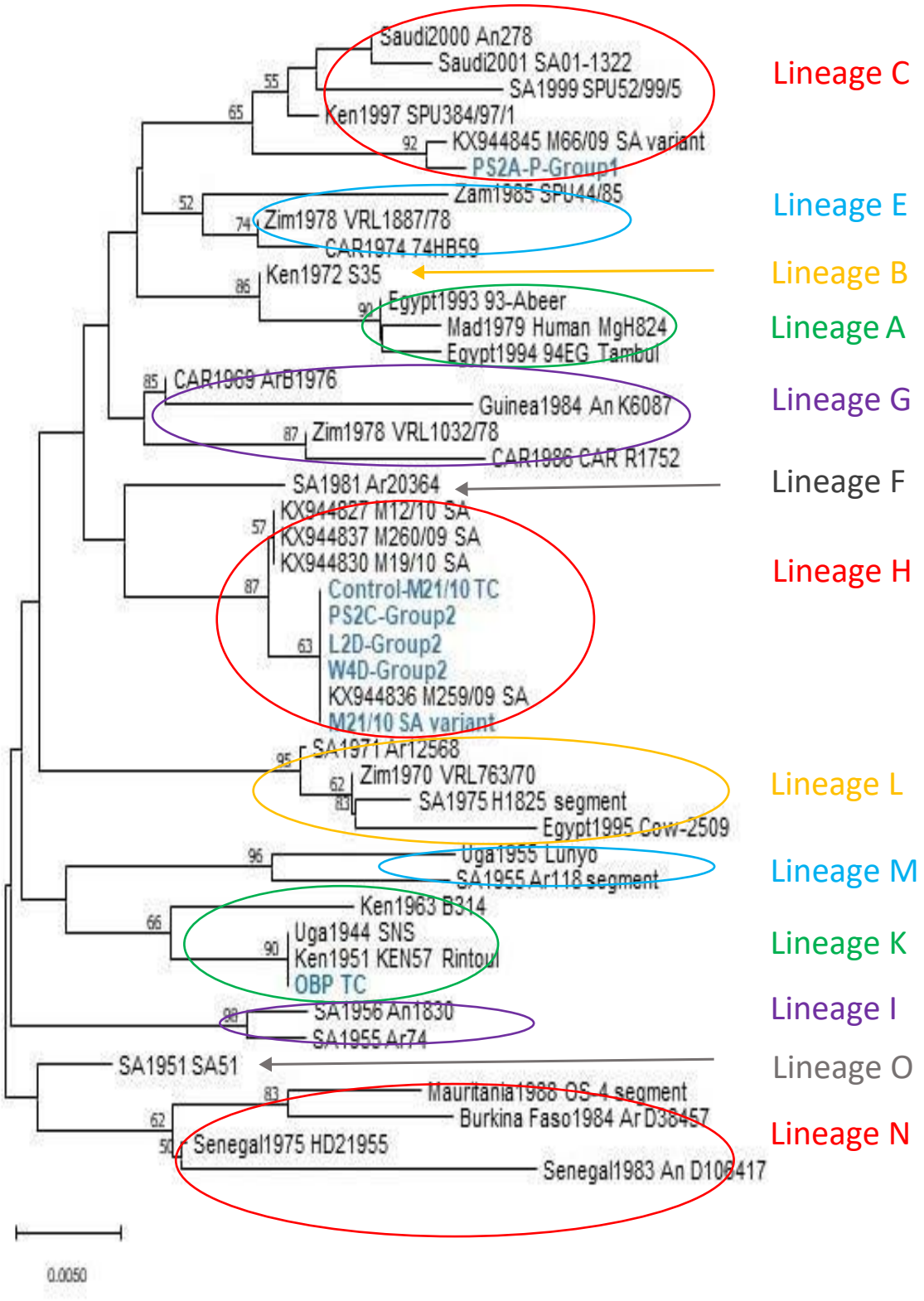

Figure 2.8 Neighbour - Joining tree showing clustering based on partial Gn glycoprotein sequences generated for the C, H and K lineage viruses characterised in this study (taxa indicated in blue and bold). Nucleotide distances were computed using the Maximum Composite Likelihood method and nodal support was tested through 10 000 non-parametric bootstrap replications. Bootstrap values above 50 are shown above the branches.

2.3.7 Electron microscopy

Round to icosahedral particles of 90nm - 110nm consistent with the shape and size of RVFV were identified by negative staining of liver samples of aborted foetuses (n = 3) from a sow infected with the RVFV M66/09 variant (Fig. 9).

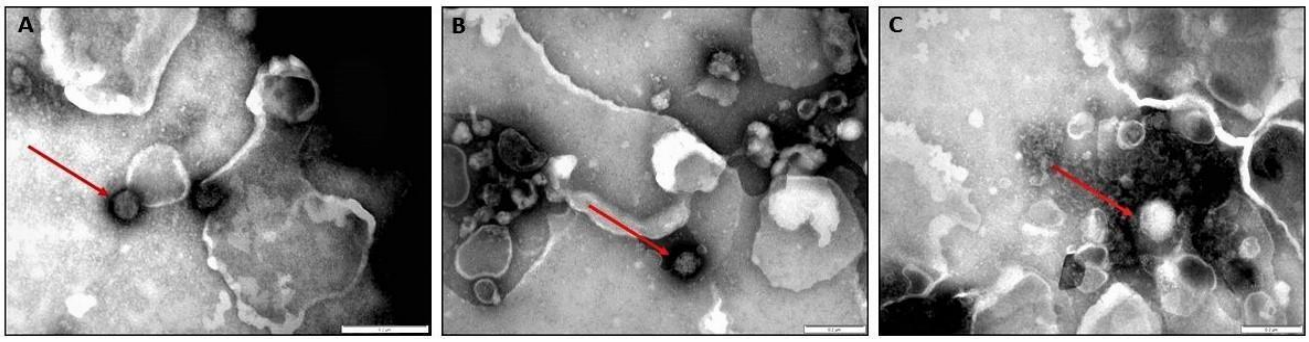

Figure 2.9 A, B and C: Negative staining electron microscopic images of livers of three foetuses showing putative RVF virions of approximately 100-120nm (denoted with red arrows). The image scale is 200nm

2.4. Discussion

Successful experimental infection of pregnant sows with RVFV was achieved in this study. Sero-conversion of 5/5 (100%) and 2/4 (50%) of sows infected with two genetically diverse RVF viruses was demonstrated (Fig. 5). Real time RT-PCR testing of organ pool samples of all the sows and their blood did not yield positive results, but oronasorectal swab pools confirmed the presence of RVFV RNA in two pregnant sows in group 1, and one in group 2. In addition, RVFV antigen/RNA, and antibodies, were demonstrated in livers, organ pools and blood, and sera of the offspring of the RVFV infected sows respectively, attesting to successful infection of their dams since they were not inoculated (Fig. 4 and 5; Appendix 2 and 3).

Suckling piglets and weaners were also successfully inoculated with RVFV in this study, as shown by seroconversion in ELISA, and demonstration of viral antigen in their livers, and RNA in their organ pools, blood and oronasorectal swabs, using immunohistochemistry and real time RT-PCR respectively (Fig. 4 and 5; Appendix 2, 3 and 4). These findings corroborated those of Clarke et al. (2021) who infected six weaners with 10^5 pfu/ml of RVFV ZH501 strain subcutaneously, and demonstrated that whist all seroconverted from 5 DPI onwards, RNA could not be directly detected from their sera and organs. In Clarke et al. (2021)'s study, viral genomic material was indirectly detected in sera (n = 3) collected on DPI 1 and 2, and oronasal swabs (n = 2) collected on DPI 3 and 5, following isolation in tissue culture (Mean Ct-value: 31.15). The differences in the proportion of samples positive for antibody in sera and RNA in oronasal swabs between this and Clarke et al. (2021)'s study, were significant ($P < 0.05$) (Appendix 5).

Nucleotide sequences obtained from porcine and ovine samples infected with the two distinct virus strains (M66/09 and M21/10) were shown to cluster within the lineages of the infecting virus strains (Fig. 8), further confirming successful infection of the pigs with RVFV.

Effect on reproduction:

Reproductive failures characterised by an abortion and expulsion of normal, macerated and mummified foetuses, birth of stillborn and weak piglets, and neonatal mortalities, were observed. The live piglets tested RVFV antibody, antigen/RNA positive in various samples, while the aborted foetuses also tested positive for viral RNA in a few samples (Table 2; Fig. 1, 4 and 5; Appendix 2 and 2.3). Reproductive failures like these may result from non-infectious or infectious causes and their resultant pathogenesis, or both (Pozzi & Alborali, 2012). However, RVFV was the most likely cause because common infectious pathogens associated with stillbirth, mummies, embryonal deaths and infertility (SMEDI) were unlikely since the pigs were sourced from a closed breeding herd with strict biosecurity and adherence to disease control regulations, conditions which are also protective against management causes. Our experimental findings support Weiss's field observations (Weiss, 1957) that pregnant sows aborted amidst ewe abortions during an outbreak of RVF in South Africa in the 1950s.

Vertical transmission of RVFV occurred as evidenced by presence of anti-RVFV antibodies in the sera of newborn piglets and through detection of viral RNA in their organ pools and blood samples. The RVFV-positive newborn piglets were from the sows that farrowed in groups 1 and 2 (Fig. 5; Appendix 2 and 3). The following findings provided further proof of vertical transmission of RVFV from sows to their offspring: i). presence of viral genomic material in an organ pool of one aborted foetus; ii). demonstration of putative RVFV particles by negative staining electron microscopy in liver samples of three foetuses in group 1; iii). positive antigen labelling in IHC sections of livers of aborted foetuses and new-born piglets from the two groups, albeit faint due to the low concentrations of virus in the tissues as evidenced by high PCR Ct-values, (Table 4; Fig.4, Fig. 9; Appendix 2 and 3). These observations also provided proof that the abortion in this study was caused by infection with RVFV. Nonetheless, vertical transmission of RVFV without demonstration of viraemia, clinical signs and seroconversion in dams and offspring does occur, as proven by Antonis et al. (2013) who demonstrated presence of viral RNA in pregnant ewes and their foetal organs, and live virus in the organs of the foetuses, similar to group 2's PS 1 and PS 4 and their offspring (Appendix 2 and 3).

Teratogenicity in piggeries, caused by hereditary factors, nutritional, or poisons and infectious agents, is a common occurrence worldwide and incidence rates of 0.11% to 4.96% have been reported (Straw et al., 2009). In this study, congenital defects in the aborted foetuses and newborns were observed in 9% of piglets (Table 2; Fig. 1). Coetzer (1980) reported that mouse brain passaged

and live-attenuated Smithburn vaccine strains caused abortions and teratogenic effects, including arthrogryposis, at 42 to 74 days of pregnancy in ewes (Coetzer & Barnard, 1977). It is therefore possible that RVFV was the cause of some of the congenital abnormalities observed in the pigs, but the phenomenon involving non-vaccine strains needs further investigation (Stedman et al., 2019). No evidence of teratogenicity was found in naturally infected ovine foetuses (Odendal et al., 2020b).

Serology:

Seropositive pigs were observed, but not all infected pregnant sows, suckling piglets and weaners, and newborn piglets tested positive for RVFV antibodies using the IDVET RVF Blocking ELISA kit (Fig. 5; Appendix 2 and 3). The negative serology results for the pigs could have been due to deposition of the virus in subcutaneous tissue instead of inside the jugular vein (Poland et al., 1997); virus replication failure in the infected pigs (Daubney et al., 1931); dominance of cell mediated, instead of a humoral immune response (Escribano et al., 2013); absence of anti-RVFV antibodies in the colostrum and milk of the sows, thus no absorption of the antibodies by offspring; failure of piglets to suckle from sero-positive sows; failure of the virus to cross the placenta and infect all foetuses (Jungersen et al., 2001); and development of immune tolerance by the infected foetuses. Alternatively, it could point to low levels of sensitivity of the test. Control lambs infected with M21/10 virus variant tested negative for antibodies, probably because they were euthanised on 3 DPI before mounting measurable immune responses. However, control lambs infected with M66/09 virus variant only tested positive on 29 DPI. These combined results suggest that the cause of the majority of the negative results in both pigs and lambs is likely due to low sensitivity of the ELISA kit used in this study (Comtet et al., 2010; Lubisi et al., 2019).

Polymerase Chain Reaction:

Presence of viral genomic material was demonstrated in some, but not all organ pools, oronasorectal swabs and blood of infected animals and their offspring by real-time RT-PCR (Appendix 2 and 3). The mandatory inactivation protocols and movement of samples from the BSL3 stable facility to the diagnostic laboratory resulted in unavoidable suboptimal sample storage conditions. In addition, the time lapse before testing (Hedman & Radstrom, 2013; Radstrom et al., 2003; Zhan et al., 2012); assay validation in porcine samples and reagents used (Fleige & Pfaffl, 2006; Zhan et al., 2012); sample pooling (Frosth et al., 2017; Muniesa et al., 2014); and presence of virus below the assay's minimum detection range (Clarke et al., 2021), could have contributed to some samples testing negative. Sample pooling most likely reduced assay sensitivity due to the dilution effect (Frosth et al., 2017; Muniesa et al., 2014). The pathogenesis of RVFV in the pig model has never been extensively studied and undisseminated infection in some of the inoculated pigs, gestation period at the time of infection of the pregnant sows and failure of the virus to cross the placenta of some foetuses cannot be ruled out (Mengeling et al., 1994).

Routes of transmission:

It was interesting that several oronasorectal swab pools from pigs (58.8%) in this study yielded positive results on PCR, highlighting the possibility that RVFV could be shed in the secretions and/or excretions of infected pigs (Appendix 2 and 3). Based on our results, shedding is estimated to occur for at least one month, since the oronasorectal swab pool of one infected weaner in group 1 (M66/09 virus variant) was positive at 28 DPI. However, because the swabs were pooled, it was not possible to identify which excretion/secretion *i.e* oral, nasal or rectal, contained the viral RNA. This, and the lack of virus isolation from these swabs is a limitation of this study. Nonetheless the results were consistent with those of other studies which reported positive RVFV PCR results from oral and nasal swabs of experimentally infected animals or isolation of virus from such samples, or both (Clarke et al., 2021; Faburay et al., 2016; Wichgers Schreur et al., 2016).

Contact transmission of RVFV via an unknown route under experimental conditions on the 7th day post exposure was first observed by Harrington et al. (1980). Busquets et al. (2010) later described transmission of RVFV from lamb to lamb though an unclear mechanism. Clarke et al. (2021) and Wichgers Schreur et al. (2016) did not record horizontal transmission even though virus was present in the oronasal and saliva swabs of infected animals respectively. In our study, viral RNA was present in the organ pools of a negative control suckling piglet (group 1), two lactating ewes and sows each (group 2) and a weaner (group 3), and from the swab of a weaner (group 2) collected five days post exposure (DPE) (Appendix2 and 2.3). Anti-RVFV antibodies were demonstrated in controls, lactating sow and weaner on 14 and 21 DPE in group 1, and one weaner each in groups 2 and 3, on 14 and 30 DPE respectively (Appendix 2 and 3). Contact with the infected secretions could have been the mode of transmission to the mock infected and uninfected animals. However, the combined results of our study and those conducted previously under experimental conditions, showed that RVFV can be transmitted horizontally among in-contact animals, even though the exact mechanism of transmission is not known.

Virus isolation:

We attempted to isolate RVFV from organ pools and terminal bleeds of the lactating and pregnant sows, aborted foetuses, newborn piglets, suckling piglets and weaners using Vero cell lines in this study. Similar to the PCR results, factors such as sample pooling, processing, storage, handling and time lapsed before testing, and the fact that RVFV infection kinetics in the pig model are unknown, could have affected the success rate of isolating virus from the majority of the samples. Consistent cell degenerative changes characterised by non-lytic cell swelling were, however, observed for a number of organ pool samples, and positive PCR results were obtained from some of the corresponding TC supernatants following two to three blind passages, suggesting some degree of virus replication (Fig. 6; Appendix 2 and 3).

Genetic variation:

Genetic analysis of the TC grown and passaged viruses, and one organ pool swab virus in this study, revealed that they clustered within three of the 15 different lineages identified by Grobbelaar et al. (2011), *viz*. lineage C, H and K (Fig. 8). Viruses from a weaner, pregnant sow and control lamb in group 2 (infected with M21/10 virus variant), clustered within lineage H with strain M21/10. The OBP virus grouped with strains in lineage K, while a piglet born to a pregnant sow from group 1 (infected with M66/09 virus variant), clustered with strain M66/09 in lineage C.

In a previous study, full genome sequences of strains M66/09 and M21/10 at passage levels 1 to 3BHK each, confirmed the M-segment clustering of these viruses within lineage C and H, respectively (Maluleke et al., 2019). In this study, the viruses were further passaged in Vero cells to increase their titres before inoculating the animals, and except for one weaner sample from group 2, additional passages in Vero cells following termination of the animal experiments were done before sequencing. The observed 0.29% and 0.86% differences in identities at nucleotide and amino acid levels respectively, between the parental M66/09 sequence deposited in GenBank and that obtained from a newborn piglet in group 1 could be attributable to mutations arising during viral replication in the different host systems (Andino & Domingo, 2015; Domingo et al., 1978; Maluleke et al., 2019), and the fact that only 353 bp partial genome sequences of the Gn glycoprotein were used to compare the isolates (Dudas & Bedford, 2019). Increased number of sequences from each infection group could have been useful in analysing clustering of viruses within the lineages, especially their relationship with parental strains, M66/09 and M21/10. Nonetheless, nucleotide percent identity differences of 0% to 5% were observed among the virus sequences used to infer phylogeny in this study, which were similar to differences observed by other workers (Gaudreault et al., 2019), further underscoring the conserved nature of the RVFV genome.

Pathology:

Numerous publications have reported on the pathology of natural or experimental RVFV infections in domestic ruminants, especially sheep, where liver friability, congestion and haemorrhage, and yellow/orange-brown discolouration due to diffuse necrosis, or disseminated grey-white areas of necrosis were the predominant findings in neonates. Other organ systems also show signs of circulatory impairment (Coetzer, 1977, 1982; Odendaal et al., 2019, 2020a, 2020b). Unlike in some adult ruminants, no gross post mortal changes were seen in the infected sows. However, in the aborted foetuses and newborn piglets, suckling piglets and weaners, macroscopic lesions similar to those in affected ruminants but with less severity, were observed mainly in the liver, spleen and kidneys. Blood-tinged hydrothorax, hydropericardium and ascites were seen in a few cases, especially in the aborted foetuses and sick newborn piglets, and similar observations were made in

sheep foetuses and lambs (Odendaal et al., 2020a, 2020b). Clarke et al. (2021) observed no lesions at necropsy, except for a slightly enlarged lymphnode on the inoculation side in one weaner.

Histopathological examinations clearly showed that RVFV infection in the ruminant neonate caused massive hepatic necrosis and haemorrhages with fatty metamorphosis and hydropic degeneration only observed among the few surviving hepatocytes (Coetzer, 1977, 1982; Odendaal et al., 2019, 2020a, 2020b). The insult to the porcine neonate liver was mainly characterised by hydropic degeneration (cellular swelling/hydropic change/vacuolar degeneration/cellular oedema). Another contrast observed was in the kidney and spleen, where subcapsular haemorrhages featured prominently in ruminants compared to mainly congestion in the pig samples. In addition, there were striking differences observed in the spleens where lymphocytolysis was a prominent lesion mostly in the red pulp of foetuses and lambs, and white and red pulp of adult sheep, while piglets and weaners showed white pulp expansion (Table 4) (Odendaal et al., 2019, 2020a, 2020b). However, tubular epithelial injury without meaningful inflammation, with or without proximal tubular epithelial (PTE) cell degeneration with pyknotic nuclei and detachment of cells from the tubular basement membrane, was a common kidney lesion between the ovines and porcines (Odendaal et al., 2019, 2020a, 2020b). The only microscopic lesions reported by Clarke et al. (2021) were mild lymphoplasmacytic perivascular cuffing and multifocal glial nodules with vacuolation in the brain neuropils of two viraemic weaners. The development of non-lipid, glycogen filled vacuoles in the hepatocytes of the infected porcine livers could underlie the apparent tolerance to infection and prevention of degenerative changes and necrosis (Damjanov, 2009; Nayak et al. (1996).

Summary and conclusion:

There were clear similarities and differences in the clinico-pathological outcome of RVFV infection in the domestic pig and sheep and cattle observed in this, and other studies. Similarities were that pregnant animals aborted, virus was vertically transmitted, reproductive disorders occurred, anti-RVFV antibodies and viral RNA could be detected in offspring born from infected sows, that sub-adult and non-pregnant animals did not display clinical signs, and that macroscopic lesions characteristic of RVFV infection were notable in the liver, spleen and kidneys. Inconsistencies with clinico-pathological outcomes and laboratory analysis of samples from experimentally infected animals characterised by negative results for several, but one or two analytes, were common among this study and others conducted in pigs, sheep and rats. Differences were that neonatal piglets were sub-clinically infected, unlike their domestic ruminant counterparts, and on histopathology, liver lesions in infected pigs were mainly characterised by mild necrosis and non-lipid glycogen-filled vacuoles. This is contrary to severe pan-necrosis observed in other domestic ruminant species.

It is concluded that domestic pigs can be infected with very high RVFV titres *via* a yet to be determined efficient route and their oronasal secretions potentially act as brief sources of virus to close in-contact susceptible animals. The blood of infected newborn piglets and weaners can also potentially infect open human skin and wounds. It is advisable that personal protective equipment (PPE), just like with ruminants, should be used when slaughtering, assisting with farrowing related processes, and handling/performing post mortem examinations on aborted foetuses and carcasses of pigs during RVF outbreaks, in order to prevent possible pig to human transmission of the disease.

CHAPTER 3

EVALUATION OF A VIRUS NEUTRALISATION TEST FOR DETECTION OF RIFT VALLEY FEVER ANTIBODIES IN SUID SERA

Abstract

Rift Valley fever (RVF) is a vector-borne viral disease of ruminants mainly, and man, characterised by abortions and neonatal deaths in animals and flu-like to more severe symptoms that can result in death in humans. The disease is endemic in Africa, Saudi Arabia and Yemen, and outbreaks occur following proliferation of RVF virus (RVFV) infected mosquito vectors. Vertebrate animal maintenance hosts of RVFV, which serve as a source of virus during inter-epidemic periods remain unknown, with wild and domestic suids being largely overlooked. To address this, we evaluated the virus neutralization test (VNT) for RVF antibody detection in suid sera, as a first step in assessing the role of suids in the epidemiology of RVF in Africa. Testing of experimental and field sera from domestic pigs and warthogs with a commercial RVF competitive antibody ELISA served as a reference standard against which the VNT results were compared. Results indicate that VNT can detect anti-RVFV antibodies within three days post-infection, has an analytical specificity of 100% and diagnostic sensitivity and specificity of 80% and 97%, respectively. Although labourintensive and time-consuming, the VNT proved suitable for screening suid sera and plasma for presence of RVFV antibodies in viraemic and recovered animals.

Keywords: Rift Valley fever; Rift valley fever virus; Inter-epidemic period; domestic pig; ELISA and VNT

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3.1 Introduction

Rift Valley fever (RVF) is a vector-borne disease of ruminants, camels and man, characterised by widespread abortions, teratogenicity, and neonatal deaths in animals, and flu-like symptoms which can progress to severe disease and even death in humans (Daubney et al., 1931; Ikegami & Makino, 2011). The causal agent is RVF virus (RVFV), genus *Phlebovirus*, order Bunyavirales, and family Phenuiviridae (Maes et al., 2018). The disease is endemic in Africa, Saudi Arabia and Yemen, and mosquitoes, primarily those of the *Aedes* and *Culex* genera, act as vectors and transmit RVFV from one host to the other. Unborn animal foetuses can contract infection transplacentally (Coetzer, 1980) and vertical transmission in humans has also been reported (Adam & Karsany, 2008; Arishi et al., 2006). Outbreaks occur after periods of high rainfall or in environments supporting the proliferation of RVFV-infected mosquito vectors (Balenghien et al., 2013). Up until recently, it was believed that transovarial transmission of RVFV by infected *Aedes* mosquitoes allowed them to act as interepidemic period (IEP) reservoir hosts but current research indicates that whilst vertical transmission in mosquitoes is likely, there is insufficient evidence to support the hypothesis (Linthicum et al., 1985; Lumley et al., 2017). Serological evidence of lowlevel circulation of RVFV among wild and domestic animals during the IEP exists, but definitive mammalian reservoir hosts remain unidentified (Mbotha et al., 2018).

Due to its zoonotic nature, a “One health approach” involving a wide range of institutions and authorities with different expertise is usually adopted in response to outbreaks and when instituting preventative measures. Diagnosis of RVF employs various serological and agent identification methods such as antibody detecting ELISAs, as well as VI, VNT, indirect immunofluorescent assay (IIFA), IHC, and RT-PCR (OIE, 2018a). Selection of an assay depends on the timing of sampling relative to suspected disease onset and availability of an assay of interest. A combination of molecular and serological assays is ideal for disease confirmation when onset of infection is unknown (Petrova et al., 2020). However, molecular tests such as RT-PCR which detect genomic nucleic acids of the infectious agent are expensive and most useful during viraemia and approximately 8 days following appearance of clinical signs for confirmation of infection with RVFV (Petrova et al., 2020). They also require expensive and sophisticated equipment, and highly skilled personnel to perform analysis, thus making relatively less expensive and simpler serology based assays more attractive in resource-poor laboratories (Abou Tayoun et al., 2014).

Serology is the scientific study of serum and other body fluids. Even though analytes that can be detected in serum and other body fluids include antibodies, disease agents such as viruses and bacteria, antigens, nucleic acids and non-antigenic compounds, serology generally implies detection of antibodies in serum or blood plasma in diagnostic laboratories.

Antibodies are formed in response to infection with microorganisms, or foreign or in the case of autoimmune disorders, own proteins. Demonstration of presence of antibodies to a pathogen provides useful information such as proof of exposure of an animal or human to the particular pathogen or vaccination against it, extent to which a disease has spread, vaccine coverage or changes in disease epidemiological trends such as reaching previously uninfected areas, affecting more animals than before or switching hosts (OIE, 2018a). Depending on their planned use, serological tests have to be fit for the intended purpose, thus require validation. A validated serological assay consistently yields positive or negative results for antibodies that accurately predict past or present infection status of a host with predetermined statistical certainty (Jacobson, 1998).

While the search for IEP vertebrate maintenance hosts of RVFV for purposes of improved disease surveillance and control continues, it is imperative that the diagnostic tools used to analyse samples from species other than domestic ruminants are confirmed fit for the intended purposes. This was highlighted in chapter 2 when the commercial IDVet blocking ELISA classified sera from RVFV infected pigs and piglets born from infected sows as suspect or negative, which was inconsistent with their infection statuses and histories, proving the importance of assay validation according to species of intended use. The role played by domestic and wild pigs in the epidemiology of Rift valley fever (RVF) in Africa is unknown. Evidence of seroconversion of South African domestic and wild pigs to RVF virus (RVFV) using an affordable, accurate and reliable serological test would provide proof of their exposure to the virus and infection, and thus justify further investigations. This study was therefore aimed at evaluating the VNT as a serological assay capable of detecting RVFV antibodies in domestic and wild pig sera and plasma, for implementation in a broader study investigating the potential role of suids in RVF epidemiology in Africa.

3.2 Materials and Methods

3.2.1 Viruses

Variants of two genetically distinct RVFV strains from the 2009 (M66/09) and 2010 (M21/10) outbreaks, in Gauteng and Free State Provinces of South Africa respectively, were used to inoculate pigs and control lambs in viral infectivity studies conducted at the Agricultural Research Council - Onderstepoort Veterinary Research (ARC – OVR)'s BSL 3 facility (Chapter 2). The 2009 strain was isolated from bovine liver, passaged 5 and 15 times in BHK and Vero cells respectively, and used at a titre of 5×10^6 PFU/100µL, while the 2010 virus was obtained from an ovine organ pool, passaged 3 and 14 times in BHK and Vero cells respectively, and utilised at a titre of 0.5×10^4 PFU/100µL.

3.2.2 Animal Inoculations

All animal experiments were carried out using protocols approved by the ARC-OVR's Animal Ethics Committee (AEC) under application number AEC 10.16 and endorsed by the University of Pretoria's AEC (Ref. EC057-17), and are described in full in Chapter 2.

Briefly, pregnant and lactating sows, 1 to 2-day old suckling piglets and weaner piglets were procured from the ARC – Animal Production Institute (ARC-API) and housed at the ARC - OVR - BSL 3 experimental animal facility at Onderstepoort. The animals were divided into 2 groups. Group 1 consisted of 1 lactating and 5 pregnant sows, 10 suckling piglets and 9 weaners (n = 25), and group 2 constituted 4 pregnant and 2 lactating sows, 20 suckling piglets and 9 weaners (n = 35). The variants of viruses M66/09.5BHK.15Vero and M21/10.3BHK.14Vero at their respective titres in 2 ml volumes were used to inoculate pregnant sows, suckling piglets and weaners of each group via the jugular vein.

Two suckling lambs of approximately 4 days were challenged in each group and used as controls. The animals were bled from the jugular vein using vacutainer tubes and standard methods from 0 - 7 days post infection (dpi), and at 14, 21, 28, 30 and 61 dpi. Newly born piglets were also bled. All sera (n=1352) were stored at 4 degrees Celsius until further use.

3.2.3 Sera

All sera used in the study were screened for RVFV antibodies with a commercial competitive ELISA (ID Screen®) which has multispecies application. Since RVF is not a pig disease and sources of known positive sera were unavailable, porcine sera produced in the above-mentioned experiments and field porcine and warthog samples were used for evaluation of analytical sensitivity (ASe), analytical specificity (ASp), diagnosic sensitivity (DSe) and diagnostic specificity (DSp). Crossreactivity studies could not be conducted due to lack of other *Bunyaviruses* and corresponding antisera (Table 3.1).

Table 3.1 Experimental and field samples categorised as positive and negative by the competition ELISA and used in the evaluation of the VNT. The samples used for determination of analytical performance were included for assessment of the assay's diagnostic capability

Purpose	Animals	Source	Antibody Status	Days Post Infection	Number Animals: Number of Samples
Analytical specificity	Sows	Experimental	Negative	Preinfection	11:11
	Weaners	Experimental	Negative	Preinfection	27:27
	Suckling piglets	Experimental	Negative	Preinfection	20:20
		Total			58:58
Analytical sensitivity	Weaners	Experimental: Infected with M21/10 RVFVv	Positive	3, 7, 14, 21 and 28	5:5
	Newborn piglets	Experimental: Dams infected with M21/10 and M66/09 RVFVv	Positive	22, 28, 32, and 44	4:4
	Sow	Experimental: Infected with M66/09 RVFVv	Positive	61	1:1
		Total			10:10
Diagnostic sensitivity	Suckling piglets	Experimental: Infected with M21/10 and M66/09 RVFVv	Positive	14	2:2
	Newborn piglets	Experimental: Dams infected with M21/10 and M66/09 RVFVv	Positive	22, 23, 28, 32, 44	41:41
	Weaners	Experimental: Infected with M21/10 and M66/09 RVFVv	Positive	3, 7, 14, 21, 27, 28, 30	16:30
	Sows	Experimental: Infected with M21/10 and M66/09 RVFVv	Positive	14, 21, 27, 28, 61	7:16
	Porcine: various ages	Field	Positive	N/A	6:6
	Warthogs: Mixed ages	Field	Positive	N/A	3:3
		Total			75:98

Purpose	Animals	Source	Antibody Status	Days Post Infection	Number Animals: Number of Samples
Diagnostic specificity	**S**uckling piglets	Experimental: Infected with M21/10 and M66/09 RVFVv	Negative	0– 7, 14, 21	20:132
	Newborn piglets	Experimental: Dams infected with M21/10 and M66/09 RVFVv	Negative	22, 23, 25, 32, 44	29:29
	Weaners	Experimental: Infected with M21/10 and M66/09 RVFVv	Negative	0–7, 14, 21, 28, 30	27:167
	Sows	Experimental: Infected with M21/10 and M66/09 RVFVv	Negative	0–7; 14, 21, 22, 27, 34	12:104
	Porcine: various ages	Field	Negative	N/A	725:725
	Warthogs: Mixed ages	Field	Negative	N/A	97:97
		Total			910:1254
		Grand Total			985:1352

N/A: not applicable; RVFVv: Rift valley fever virus variant

3.2.4 Additional Performance Measures

Apart from analytical and diagnostic performance, the VNT was assessed for: i) influence of potentially inhibitory factors in serum arising from haemolysis and putrefaction (n = 2); ii) repeatability, where experimental sera (n = 2) were tested in replicates of 4 in 5 plates for 7

consecutive days; iii) robustness, through first incubation of replicate test plates for different time periods (60, 90 and 120 min), using different test virus titres (100–300 and 1000 $TCID_{50}$/mL) in replicate plates, and further incubating the plates 1 - 3 days following recording of results; and iv) reproducibility where inter-analyst comparison (*n* = 84) and inter-method comparison with an inhouse IgG indirect ELISA (*n* = 119) were performed (Williams et al., 2011).

3.2.5 Serological Tests

a). ELISA

The ID Screen® Rift Valley Fever Competition Multi-species ELISA (ID-VET, Montpellier, France) intended for detection of both IgM and IgG anti-Rift Valley Fever (RVF) antibodies in ruminant serum or plasma was used as the standard of comparison for the VNT. The test is reported to have a diagnostic sensitivity and specificity of 98% and 100% respectively (Kortekaas et al., 2013).

Briefly, 50 µl of buffer was added to each well of the coated microplates, followed by 50 µl of control positive and negative sera, and test sera in their designated wells, bringing the total volume in each well to 100µl. The plates were incubated at 37^0C for hour and washed three times with wash solution. AntiRVFV – NP conjugate was added at 100µl volumes and incubated at 21^0C for 30 minutes. The plates were washed and 100µl of substrate added, followed by incubation for 15 minutes at 21^0C, following which 100µl of stop solution was added and the plates read at a wavelength of 450 nm with an ELISA microplate reader (BIOTEK ELx 808).

b). Virus Neutralisation Test

The VNT was conducted as described previously with a few modifications (Mroz et al., 2017). Test sera were heat inactivated at 56 °C for 30 min and allowed to cool. Initial 1/5 dilutions in DMEM (Lonza, Switzerland) containing non-essential amino acids (NEAA), Penicillin, Streptomycin and Ampotericin B, were loaded on 96 well plates in duplicate and subsequent two-fold dilutions made down the plates. Virus variant M66/09 5BHK.15Vero described previously was added at titres of 100–300$TCID_{50}$ to each well containing serum, and the plates were incubated at 37°C for 1 h in a humid chamber with 5% CO_2. Vero cells (ATCC, USA) at a concentration of 3 to 4 × 10^5 cells per ml were added and the plates were incubated at 37 °C for 3–5 days. Cell, virus titration, sample, and control sera plates were included with each test run, with reference ovine RVFV (strain 35/74) antiserum and serum with undetectable RVFV antibodies by in-house indirect IgG and IgM capture ELISAs used as positive and negative control sera, respectively (Williams et al., 2011).

The plates were monitored daily under an inverted microscope and when the control virus showed cytopathic effect (CPE) of 90%–100%, presence of CPE and intact cell monolayers was recorded and scored. For confirmation of results, plates were fixed with 10% formalin containing 0.05% crystal

violet, and re-visualised using the microscope. Serum antibody titres were taken as the reciprocal of the dilution at which presence of either no (0%) or minute CPE (~10%) was observed.

3.2.6 Statistics

Association, differences and agreement between the competitive ELISA and VNT were determined using Chi-square and Fisher's exact test, McNemar Chi-square, and Cohen's kappa coefficient (κ) respectively (Baveja & Aggarwal, 2017), employing GraphPad software (GraphPad Prism, 2018). Receiver operating characteristic (ROC) curve analysis was utilised to select the best VNT positive cut-off value and the graph was drawn using Microsoft Excel (Microsoft Corporation, 2016) and VassarStats Website for Statistical Computation (VassarStats, 2018). MedCalc Easy-To-Use Statistical Software (2018) was used to evaluate test performance. Confidence intervals for all calculations were set at 95%. The VNT would be deemed fit for the intended purpose if results were repeatable, reproducible and statistically and significantly in agreement with those of the competitive ELISA.

3.3 Results

3.3.1 Operating Range and Thresholds

The minimum and maximum serum dilutions at which anti-RVFV antibodies could be detected by the VNT were 1/5 to 1/1280 according to the titre of the positive control serum used. The serum dilution at which inherent toxins and haemolysis products had cleared and cell monolayer integrity and visibility was intact in more than 90% of the samples tested was at dilutions above 1/40. The best positive cut-off was set at 1/60 following ROC curve analysis and the area under the curve (AUC) was 0.94 (CI: 80%–100%) (Fig 3.1). Suspect titre was set at 1/40, which was the dilution at which known positive sera would show protection when the plates were over incubated or high testvirus titres were used.

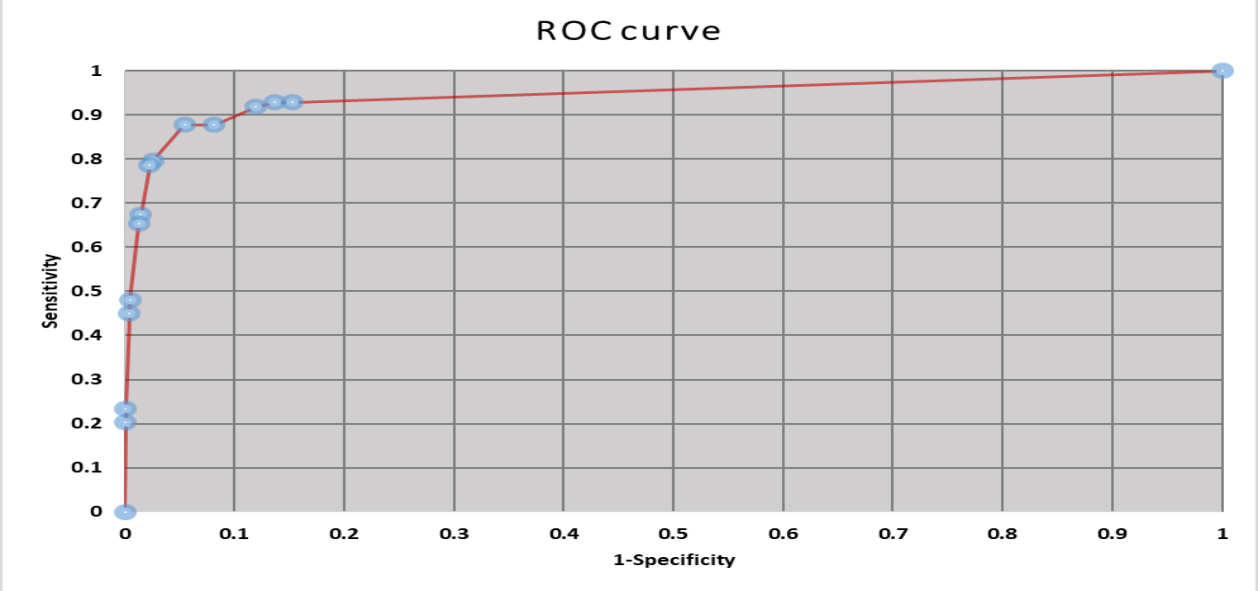


Figure 3.1 Receiver operating characteristic curve generated from 14 cut-off values, indicating the best positive cut off values to be between 1/60 and 1/80 dilutions. The area under the curve is 0.94

3.3.2 Analytical Sensitivity and Specificity

Anti-RVFV antibodies could be detected in swine sera as early as 3 dpi by both ELISA and VNT. Positive results were obtained at different dilutions according to the antibody titres of the sera tested, and 1/640 was the lowest dilution at which positives were recorded. All pre-inoculation samples tested negative with both ELISA and VNT, thus analytical specificity (ASp) was 100% (CI: 92%–100%).

3.3.3 Diagnostic Sensitivity and Specificity

For purposes of calculating diagnostic sensitivity (DSe) and diagnostic specificity (DSp), the IDVET competitive ELISA suspect results were regarded as positive and those of the VNT were taken as negative. Diagnostic sensitivity and specificity were 80% (CI: 70% to 87%) and 97% (CI: 96% to 98%), respectively

3.3.4 Additional Performance Measures

Putrefied sera and those with products of haemolysis were cytotoxic and resulted in cell lysis and unclear visibility of the cell monolayers. Intra-plate and inter-plate variability measured as percent coefficient of variation (%CV) ranged from 0%–30% and 5.7%–30% respectively, with inter-plate variability increasing above 30% only when incubation times were extended past 3–5 days and test virus titres differed by one log between plates. The %CV of the in-house indirect ELISA and VNT, and VNT inter-analyst results were 1.8% and 2.4%, and 2.8% and 3%, respectively.

3.3.5 Statistics and Fitness for Purpose

Analysis of association between the results of the ELISA and VNT using Chi-square and Fisher's exact test yielded a two-tailed *p*-value < 0.0001. A total of 52 insignificant (p = 0.12) discordant

results were discerned by the McNemar test, and the odds ratio was 1.600 (CI: 0.89 to 2.95). Number of agreements between the test classifications were 1300 (96.15%) and Kappa was 0.78 (CI: 0.66 – 0.8).

3.4 Discussion

In the absence of validated serological tests for use in non-target species, highly specific neutralisation tests are employed instead. The neutralisation method currently listed in the RVF chapter of the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the Office International des Epizooties (OIE) is the plaque reduction neutralisation test (PRNT) (OIE, 2018a).

The PRNT method allows the infective virus to cause CPE slowly and is thus ideal for quantifying the starting virus (Baer & Kehn-Hall, 2014). However, the method is more time-consuming, expensive and labour-intensive than the VNT (Di Gennaro et al., 2014). Some of the drawbacks include: i) fewer samples per plate can be tested compared to the VNT and larger sample volumes, which may not be available, are required; ii) cell-seeding and addition of dyes prior to commencement of the test and reading of the plates respectively, are needed, which adds two additional days to the test procedure; iii) gel-based medium overlays such as agarose require heating and can cause damage to cell monolayers and negatively affect heat-labile viruses if not kept at optimum temperatures; iv) dyes such as neutral red are photosensitive, have a short shelf life and can crystallize and interfere with the test; and v) because many virus strains have pinpointsized plaque phenotypes, result interpretation is difficult and must be performed by highly trained personnel (Di Gennaro et al., 2014).

While PRNT is sensitive and highly specific, it is not ideal for resource-poor laboratories whose primary intentions are to screen large numbers of field sera for RVFV antibodies following vaccination campaigns, when disease incursion is suspected or for surveillance during IEP. Since various forms of neutralisation assays are regarded as the “gold standard” when evaluating or validating serological assays, another method with high DSe and DSp was required when evaluating the suitability of VNT for RVFV antibody detection in suid samples. The ID Screen® RVF antibody competitive ELISA (ID-VET, Montpellier, France) was selected because it has a multispecies application, is reasonably validated, widely used and commercially available (Kortekaas et al., 2013; Métras et al., 2017; Monaco et al., 2015; Williams et al., 2011).

As previously hypothesized, domestic pigs can be successfully infected with high doses of RVFV (Scott, 1963), sero-convert, shed virus in their secretions and vertically transmit the virus to their offspring in-utero, as attested by demonstration of RVFV antibody, RNA, antigen, virions, and viable virus in tissue samples of sows, piglets and offspring of experimentally infected sows (Chapter 2). Due to unavailability of positive field samples, suid sera from the experimental infections had to be

utilised instead. A limitation to the study was that experimental sera collected 0 to 7 dpi were only analysed for antibody presence omitting analysis by PCR to confirm successful infection, due to limited sample volume. In addition, the number of experimental animals was out of necessity kept at a minimum with samples collected from individual pigs on different days being used to assess diagnostic performance, thus impacting sample independence. We were nonetheless able to recover an association between the outcomes of the ELISA and VNT. Agreement between the sample status classification (96.15%) was statistically significant (p <0.0001) and strong (K = 0.78), and the observed differences (3.85%) were regarded as insignificant. Repeatability and reproducibility of the VNT was good at %CV between 0%–30% and an AUC above 0.9 confirmed the robustness of the test for dichotomous discrimination of samples.

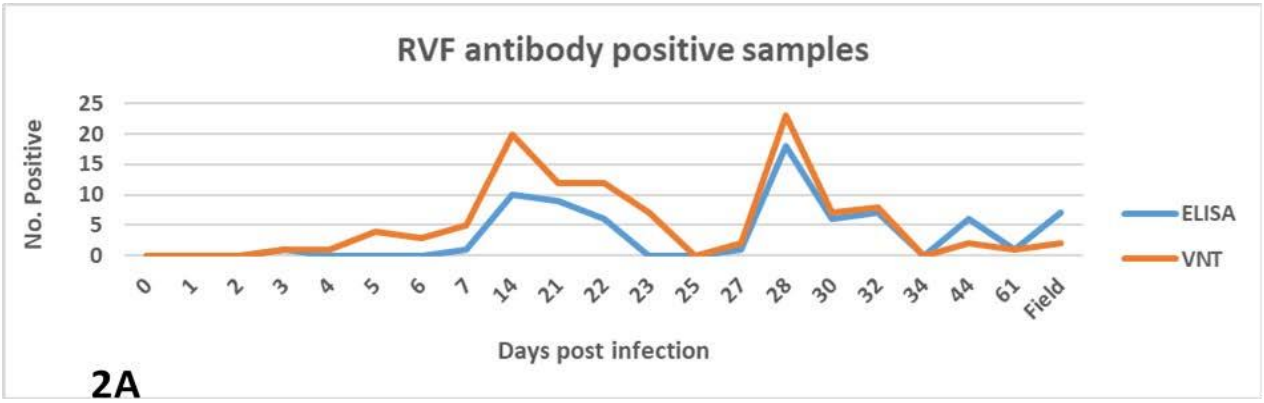
Assessment of the two tests without regarding either as the standard of comparison and taking into consideration their respective cut-off values, showed that the VNT classified more samples as positive and suspect than the ELISA (Fig 3.2), and that the level of agreement of the results (92%) was high (Table 3.2). The competitive ELISA and VNT are purported to be capable of detecting both RVFV IgM and IgG antibodies, with the IgM usually appearing within the first week of infection (Pepin et al., 2010). For the experimentally infected animals, the VNT yielded suspect (n = 34) and positive (n = 14) results from 3 to 7 dpi, while the ELISA only yielded two positive (one each on 3 and 7 dpi), and no suspect results. The majority of positive results were detected from 14 dpi for both methods. Only 1/9 ELISA-positive field sera were designated as such by the VNT, probably due to low titres.

Table 3.2 Comparison of the level of agreement between the competition ELISA and VNT in Rift valley fever (RVF) antibody status classification of experimental and field sera

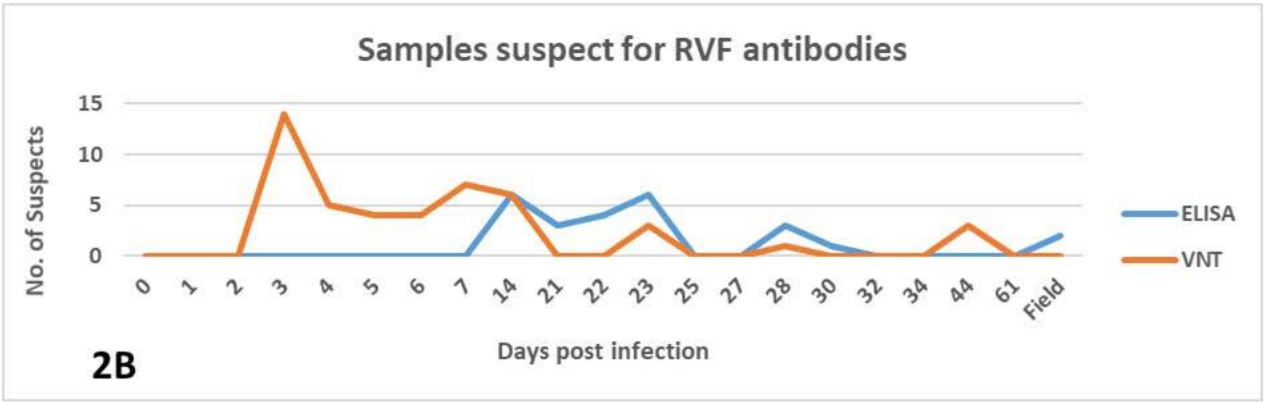
			ELISA		
		Positive	**Suspect**	**Negative**	
VNT	Positive	61 (4.5%)	17 (1.3%)	32 (2%)	110 (8%)
	Suspect	6 (0.5%)	2 (0.15%)	39(3%)	47 (3.5%)
	Negative	6 (0.5%)	6 (0.5%)	1183 (88%)	1195 (88.5%)
		73 (5.5%)	25 (2%)	1254 (93%)	1352

Suspect results would normally be re-tested using other methods or the donor animals re-bled, thus positives are unlikely to be missed. Of concern is the negative classification of several sera from experimentally infected animals between 3 and 14 dpi by the competitive ELISA, as utilisation of a method with low sensitivity for IgM antibodies would result in positive and viraemic animals being missed and in the potential for disease spread.

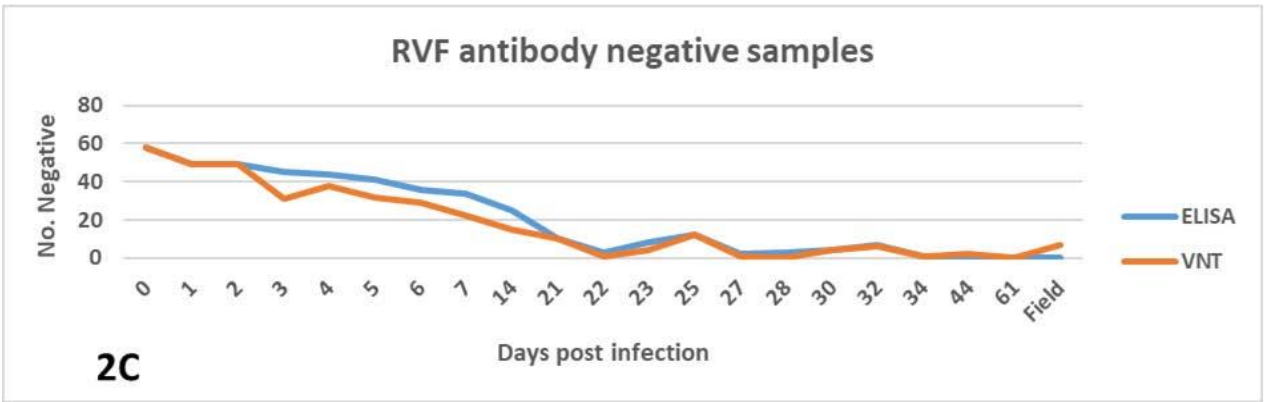
Domestic pigs are closely related to humans in terms of anatomy and physiology, and can serve as excellent animal models to study human infections (Meurens et al., 2012). In the event of using pigs to study RVF to generate new knowledge for improved animal and human health, it is imperative to evaluate the suitability of diagnostic tools for use in this species. The results of the VNT proved statistically agreeable with those of the competitive ELISA used for comparative purposes in this study. Both methods proved suitable for screening suid sera and plasma for RVFV antibodies in experimental and field studies. However, as the VNT detected more positive samples than the ELISA in experimentally infected pigs, especially during early infection, it is, in the absence of better alternatives, the preferred method for detecting RVF antibodies in suids.



2A



2B



2C

Figure 3.2A, 3.2B and 3.2C Number of RVF antibody positive, suspect and negative experimental and field sera detected by competitive ELISA and VNT. The experimental samples were constituted by sera from sows, weaners and suckling pigs, and piglets born from virus inoculated pregnant sows between dpi 0 and 61. Only discordant field sera are represented in the graphs

CHAPTER 4

SEROPREVALENCE OF RIFT VALLEY FEVER IN SOUTH AFRICAN DOMESTIC AND WILD SUIDS (1999–2016)

Abstract

Rift valley fever (RVF) is a vector-borne viral disease of domestic ruminants, camels and man, characterised by widespread abortions and neonatal deaths in animals, and flu-like symptoms, which can progress to hepatitis and encephalitis in humans. The disease is endemic in Africa, Saudi Arabia and Yemen, and outbreaks occur after periods of high rainfall, or in environments supporting the proliferation of RVF virus (RVFV) infected mosquito vectors. The domestic and wild animal maintenance hosts of RVFV, which may serve as sources of virus during inter-epidemic periods (IEPs) and contribute to occurrence of sporadic outbreaks, remain unknown, although reports indicate that the African buffalo (*Syncerus caffer*) may play a role. Due to the close proximity of the habitats of domestic pigs and warthogs to those of known domestic and wild ruminant RVFV maintenance hosts respectively, our study investigated their possible role in the epidemiology of RVF in South Africa by evaluating RVFV exposure and seroconversion in suids. A total of 107 warthog and 3,984 domestic pig sera from 2 and all 9 provinces of South Africa, respectively, were screened for presence of RVFV neutralizing antibodies using the virus neutralization test (VNT). Sero-positivity rates of 1.87% (95% CI: 0.01%–6.9%) and 0.68% (95% CI: 0.49%–1.04%) were observed for warthogs and domestic pigs, respectively, but true prevalence rates, taking test sensitivity and specificity into account, were lower for both groups. There was a strong association between the results of the two groups ($\chi 2 = 0.75$, $p = .38$), and differences in prevalence between the epidemic and IEPs were non-significant for all suid samples tested ($p > 0.05$). This study, which provides the first evidence of probable exposure and infection of South African domestic pigs and warthogs to RVFV, indicates that further investigations are warranted, to fully clarify the role of suids in the epidemiology of RVF.

Keywords domestic pig, epidemic period, inter-epidemic period, Rift valley fever, virus neutralization test (VNT), warthog

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4.1 Introduction

Rift valley fever (RVF), first reported from Kenya by Daubney et al. (1931), is a zoonotic disease which causes widespread abortions and neonatal mortalities in ruminants mainly, and flu-like symptoms that can progress to a haemorrhagic or neurological disease in humans (Ikegami & Makino, 2011). The disease is caused by RVF virus (RVFV), a *Phlebovirus* in the *Bunyavirales* order and *Phenuiviridae* family (Maes et al., 2018). Animals can acquire RVFV *in utero* from their infected dams or *via* the bites of infected mosquitoes that feed on them. Human infections are mostly associated with handling or opening infected carcasses and manipulating the virus in laboratories, and to a lesser extent, *via* mosquito bites (Davies & Martin, 2003). Many mosquito species have been proven competent vectors of RVFV naturally or by experimental means, but the *Aedes* and *Culex* species play a major role in the epidemiology of RVF. Vertical transmission of virus from female *Aedes* mosquitoes to their eggs has been reported (Linthicum et al., 1985), but some investigators argue that although possible, there is inadequate evidence to prove transovarial transmission in mosquitoes convincingly (Lumley et al., 2017).

Control of RVF relies on surveillance and early detection, laboratory confirmation, application of zoosanitary measures and vaccination of susceptible domestic ruminants (Davies & Martin, 2003). Outbreaks of disease normally occur at intervals of up to 15 years following periods of high rainfall or in environments that support the hatching of infected mosquito eggs, thus increasing the population of infected vectors (Lichoti et al., 2014; Pedro et al., 2016). Sporadic outbreaks of disease are, however, experienced and serological evidence of infection is frequently obtained during interepidemic periods (IEP) in RVF endemic areas. The source of virus in such situations is unknown (Lichoti et al., 2014; Mroz et al., 2017). Unidentified vertebrate animals and newly hatched *Aedes mcintoshi* mosquitoes are thus suspected to play a major role in the transmission cycle of virus during the IEPs (Manore & Beechler, 2015; Pedro et al., 2016).

Given the close proximity of suids to clinically susceptible ruminants in wild and domestic farming settings, studies were conducted to investigate the largely overlooked role of wild and domestic pigs in the epidemiology of RVF. Early investigations of suid involvement provided conflicting results with some studies indicating that pigs are not susceptible to RVFV, whilst others confirmed dosedependent susceptibility (Daubney et al., 1931; Easterday et al., 1962; Findlay & Daubney, 1931; Scott, 1963). More recently, RVFV antibody prevalence of 15% was reported in abattoir pigs in Egypt, with the majority of positive reactors being detected during winter, suggesting that pigs could be intermediate maintenance hosts of the virus in Egypt (Youssef, 2009). In contrast, none of the pigs evaluated in a study in Nigeria tested positive (Olaleye et al., 1996b). Anti-RVFV antibodies have also been detected in savannah warthogs (*Phacochoerus africanus*) from Kenya (Britch et al., 2013; Evans et al., 2008), but could not be demonstrated in warthogs from Zimbabwe (Anderson & Rowe, 1998). Outcomes of these studies were, however, insufficient to elucidate the role of suids in

the epidemiology of RVF (Olive et al., 2012). Rift valley fever virus persistence depends on a subtle interaction between numerous susceptible mammalian and mosquito hosts, vertical transmission and fluctuating environmental permissiveness (Manore & Beechler, 2015). It is therefore recommended that efforts to identify mammalian IEP reservoirs and understand disease dynamics should consider numerous susceptible host species and incorporate serological surveys both during epizootic periods (EP) and IEPs, and that positive reactors should be subjected to further virological testing (Britch et al., 2013; Manore & Beechler, 2015; Olive et al., 2012).

The current study aimed to investigate exposure and seroconversion of domestic pigs and warthogs to RVFV as part of a broader study to assess their possible role in the epidemiology of RVF in South Africa.

4.2 Materials and Methods

4.2.1 Study area

The study was conducted in the nine provinces of South Africa, which covers a surface area of 1,219,602 km2 and is located between latitudes 22°S–35°S and longitudes 17°E–33°E (Figure 4.1). South Africa is a relatively dry country with annual rainfall averaging 464 mm and which due to its elevation above sea level has temperatures that are lower than those of other countries at similar latitudes.

4.2.2 Sera

Domestic pig sera (*n* = 3,984) originating from the nine provinces of the country *viz*. North West (NW), Gauteng province (GP), Limpopo province (LP), Mpumalanga province (MP), Free State (FS), KwaZulu-Natal (KZN), Eastern Cape (EC), Western Cape (WC) and Northern Cape (NC), and warthog sera from the Kruger National Park (KNP) (*n* = 88) and KZN (*n* = 19) were used in the study. The domestic pig sera were retrieved from the serum bank located at the Agricultural Research Council–Onderstepoort Veterinary Research (ARC-OVR) and were submitted for sero-surveillance and diagnosis of pig diseases of economic importance (De Klerk, 2012). Five and 2 warthog sera from Pongola (KZN) and Orpen (KNP), respectively, were also sourced from this serum bank. The remaining warthog sera (*n* = 100) were secured from the KNP sample repository, with 14/100 originating from Mkhuze National Park in KZN and 86/100 from KNP (Satara: *n* = 17; Skukuza: *n* = 45; Crocodile Bridge: *n* = 1 and Marloth Park: *n* = 23) (Table 4.1). For the purpose of the current investigation, EP refers to the year a major outbreak or outbreaks occurred, irrespective of the months of occurrence or sera collection. Similarly, IEP refers to the years during which no major outbreaks were reported. South Africa experienced major RVF outbreaks between 2008 and 2011 (Métras et al., 2013), thus the 2008–2009 domestic pig sera constituted EP, whilst those of 2007 and 2012–2015 were considered IEP samples (Table 4.1). Warthog sera collected in 1999 (*n* = 1),

2006 (*n* = 3) and 2013–2016 (*n* = 94) constituted IEP material and those collected between 2008 and 2011 (*n* = 9) were classified as EP samples.

4.2.3 Serological test

The virus neutralization test (VNT) with a diagnostic sensitivity and specificity of 80% and 97% in suid sera, respectively (Chapter 3), was used as described by Lubisi et al. (2019) to detect antiRVFV antibodies in the samples. Briefly, sera were heat-inactivated at 56°C for 30 min, diluted two-fold in DMEM (Lonza) containing NEAA, penicillin, streptomycin and amphotericin B in 96 well tissue culture plates and virus at a concentration of 100–300TCID50 added to each well containing serum, followed by 1-hr incubation at 37°C and 5% CO2. Vero cells from American Type Culture Collection (ATCC) were then added at a concentration of 3 to 4 x 105 cells per ml and the plates further incubated for 3–5 days. Appropriate controls were included with every test run. Plates were viewed with an inverted microscope and CPE and cell monolayer protection scored accordingly. Plates with ambiguous results were fixed with 10% formalin and stained with 0.05% crystal violet for improved visualization. Positive and suspect cut-off values were marked by cell monolayer intactness at serum dilution of 1/60 and higher, and between 1/40 and 1/60, respectively.

4.2.4 Statistics

FreeCalc in AUSVET EpiTools (Sergeant, 2018) was used to calculate sample size for freedom testing with imperfect tests. As RVF is not considered a pig disease, prevalence (antibody presence) was presumed to be 1% and the national pig herd was estimated at 1 million (Boettiger, 2000). Since pre-collected sera were used, samples were selected on the basis of availability, quality and quantity, to ensure representation of all submitting state veterinary areas, rather than by statistical guidelines. The serological results entered in Excel spreadsheets (Microsoft Corporation, 2016) were used to calculate seroprevalence and odds ratios using MedCalc Software (MedCalc Easy—To—Use Statistical Software, 2018). Chi-square with Yates' correction and Fisher's exact test were performed to determine associations between the observed seropositivity between the provinces and sampling periods using GraphPad software (GraphPad Prism, 2018). True seroprevalence estimates were calculated using the AUSVET EpiTools epidemiological calculator (Sergeant, 2018), which uses the formula:

$$\text{True Prevalence} = (\text{Apparent Prevalence} + \text{Specificity} - 1) \,/\, (\text{Sensitivity} + \text{Specificity} - 1)$$

4.3 Results

Seroconversion of South African domestic pigs and warthogs to RVFV was demonstrated in this study. Antibody positive domestic pig sera were detected from all provinces except for GP, whilst positive warthog cases came from KNP. Overall test seroprevalences, which were regarded as apparent prevalences because the VNT used did not have 100% diagnostic sensitivity and specificity, were 0.68% (95% CI: 0.47%–0.98%) and 1.87% (95% CI: 0.51%–6.56%) for domestic

pigs and warthogs, respectively. Limpopo province had the highest overall domestic pig seropositivity at 1.9% (CI: 1.07%–3.39%). However, in 2008, 2009 and for the 2012–2015 period, the highest prevalence rates were observed for EC, LP and WC, respectively (Table 4.1). For domestic pigs, test seroprevalence rates in 2008, 2009 and 2012–2015 did not differ significantly (Table 4.1). Seropositivity for the EP and IEP were each 0.68% (CI: 0.44%–1.03% and 0.24% and 1.62%), and the odds of finding more seropositive pigs during the EP (2008 and 2009) compared to the IEP (2007 and 2012–2015) were similar (OR = 1; 95% CI: 0.378–2.654; Z-statistics = 0.004), indicating no significant differences in pig RVFV infection and seroconversion rates between the two periods.

Among the warthogs, seropositive animals were only observed during the IEP (2013–2016) (Table 4.1), and similar to domestic pigs, the test seropositivity rates between the EP (2010–2011) and IEP were statistically non-significant (OR = 1.6; 95% CI = 0.07%–37%; Z-statistics = 0.3). Differences in seroprevalence rates between the domestic pigs and warthogs were also non-significant irrespective of sampling periods, and the odds of finding more seropositive warthogs than domestic pigs in the sampled locations were negligible (OR = 2.8; 95% CI: 0.6552– 11.8930; Z-statistics = 1.38). True overall, provincial, EP and IEP seroprevalence rates for both domestic pigs and warthogs were less than zero and inconsistent with the assumed test sensitivity and specificity (Sergeant, 2018). For purposes of the epidemiological study, the estimated negative true prevalence rates were thus truncated to zero (Fig 4.1; Table 4.1).

Table 4.1 Sero-prevalence of RVFV antibodies in South African suids from all 9 provinces discerned by VNT in this study. Estimated true prevalence rates for all provinces, Kruger National Park and periods sampled were negative and truncated to zero

Domestic pigs					
Province	**2007**	**2008**	**2009**	**2012-2015**	**Total**
	No. positive/no tested	**No. positive/no tested**	**No. positive/no tested**	**No. positive/no tested**	
NW	0/0	0/68 AP: 0%. CI:0% to 5.35%	0/284 AP: 0%, CI: 0% to 1.33%	1/51 AP: 1.96%, CI:0.35% to 10.3%	1/403 AP: 0.25%, CI:<0% to 1.39%
GP	0/0	0/100 AP: 0%, CI:0% to 3.7%	0/300 AP: 0%, CI: 0% to 1.26%	0/92 AP: 0%, CI: 0% to 4.01%	0/492 AP: 0%, CI:0% to 0.77%
LP	0/0	1/145 AP: 0.69%, CI: 0.12% to 3.8%	9/380 AP: 2.37%), CI: 1.25% to 4.44%	1/50 AP: 2%, CI: 0.35% to 10.5%	11/575 AP: 1.91%, CI:1.07% to 3.39%
FS	0/0	0/92 AP: 0%, CI: 0% to 4.01%	3/184 AP:1.63%, CI: 0. 56% to 4.68%	0/99 AP: 0%, CI: 0.01% to 3.74%	3/375 AP: 0.80%, CI:0.27% to 2.33%

Domestic pigs					
Province	**2007**	**2008**	**2009**	**2012-2015**	**Total**
	No. positive/no tested	**No. positive/no tested**	**No. positive/no tested**	**No. positive/no tested**	
MP	0/0	0/152 AP: 0%, CI:0% to 2.46%	0/283 AP:0%, CI:0% to 1.34%	.26%1/48 AP:2.08%, CI: 0.37% to 10.9%	1/483 AP: 0.2%, CI:<0% to 1.16%
KZN	0/0	1/102 AP: 0.98%, CI: 0.17% to 5.35%	0/302 AP:0%; CI:0% to 1.26%	1/54 AP:1.85%, CI: 0.33% to 9.77%	2/458 AP: 0.44%, CI:0.12% to 1.58%
EC	0/0	3/128 AP: 2.34%, CI: 0.8% to 6.66%	3/303 AP: 0.99%, CI: 0.34% to 2.87%	0/104 AP:0%, CI: 0% to 3.56%	6/535 AP: 1.12%, CI:0.51% to 2.42%
WC	0/102 AP: 0%, CI: 0% to 3.63%	0/0	0/92 AP:0%, CI: 0% to 4.01%	1/46 AP: 2.17%, CI: 0.38% to 11.34%	1/240 AP: 0.42%, CI:<0% to 2.32%
NC	0/0	0/100 AP: 0%, CI: 0% to 3.7%	2/230 AP: 0.87%, CI: 0.24% to 3.11%	0/93 AP: 0%, CI:0% to 3.97%	2/423 AP: 0.47%, CI:0.13% to 1.71%

Domestic pigs					
Province	**2007**	**2008**	**2009**	**2012-2015**	**Total**
	No. positive/no tested	**No. positive/no tested**	**No. positive/no tested**	**No. positive/no tested**	
Total	0/102 AP: 0%, CI: 0% to 3.63%	5/887 AP: 0.56%, CI: 0.24% to 1.31%	17/2358 AP: 0.72%). CI: 0.45% to 1.15%	5/637 AP:0.78%, CI: 0.34% to 1.82%	27/3984 AP: 0.68%, CI:0.47% to 0.98%
Warthogs					
Province	**1999**	**2006**	**2008-2011**	**2013-2016**	**Total**
	No. positive/no tested	**No. positive/no tested**	**No. positive/no tested**	**No. positive/no tested**	
KNP	0/1	0/3	0/9	2/75	2/88
	AP: 0%	AP: 0%	AP: 0%	AP: 2.67%, CI: 0.73% to 9.21%	AP: 2.27%, CI: 0.63% TO 7.981%
KZN	N/A	N/A	N/A	0/19 AP: 0%	0/19 AP:0%
Total					2/107 AP: 1.87%, CI:0.51% to 6.56%

AP: Apparent prevalence; NW: North-West; GP: Gauteng Province; LP: Limpopo Province; FS: Free State; MP: Mpumalanga Province; KZN: Kwa-Zulu Natal;: Eastern Cape; WC: Western Cape; NC: Northern Cape

T**able 4.2** Rift valley fever sero-prevalence rates in RVFV permissive mammalian hosts other than the amplifying domestic ruminants in Africa and the Arabian Peninsula

Country	Period	Year	Animal/species	Prevalence	Reference
Botswana	DNR	2010-2011	Buffalo (*Syncerus caffer*)	12.7%	Jori et al. (2015)
Central African Republic	DNR	2010-2012	Human (*Homo sapiens*)	16.7%	Nakouné et al. (2016)
Djibouti	DNR	2010-2011	Human (*Homo sapiens*)	2.2%	Andayi et al. (2014)
Egypt	IEP	2000	Black rat *(Rattus rattus)*	29.33%	Youssef and Donia (2001)
	IEP	2007-2008	Pig (*Sus scrofa domesticus*)	15%	Youssef, 2009
			Camel *(Camelus dromedarius*)	3.17%	Mroz et al. (2017)
			Buffalo *(Bubalus bubalis)*	5.85%	Mroz et al. (2017)
Gabon	DNR	2005-2008	Human (*Homo sapiens*)	3.30%	Pourrut et al. (2010)
Kenya	IEP (pre)	1999-2006	Buffalo (*Syncerus caffer*)	15.6% and 1.2%	Britch et al. (2013); Evans et al. (2008)
			Camel *(Camelus dromedarius*)	7.1%	Britch et al. (2013)
			Eland (*Taurotragus oryx*)	0%	Britch et al. (2013); Evans et al. (2008)
			Elephant (*Loxodonta africana*)	6%	Evans et al. (2008)
			Gazelle (*Gazella*)	87.5% and 9.1%	Britch et al. (2013); Evans et al. (2008)
			Giraffe (*Giraffa*) ;	0%	Britch et al. (2013); Evans et al. (2008)

Country	Period	Year	Animal/species	Prevalence	Reference
Kenya	IEP (pre)	1999-2006	Impala (*Aepyceros melampus*)	62.5%	Evans et al. (2008)
			Kongoni (*Alcelaphus buselaphus*)	0%	Britch et al. (2013); Evans et al. (2008))
			Lesser Kudu (*Tragelaphus imberbis*)	50%	Evans et al. (2008)
			Leopard (*Panthera pardus*)	0%	Evans et al. (2008)
			Lion (*Panthera leo*)	0%	Evans et al. (2008)
			Black Rhino (*Diceros bicornis*)	32.6%	Evans et al. (2008)
			Waterbuck (*Kobus ellipsiprymnus*)	20% and 11.8%	Britch et al. (2013); Evans et al. (2008)
			Warthog (*Phacochoerus africanus*)	2.5%	Evans et al. (2008)
			Zebra (*Equus quagga*)	1%	Evans et al. (2008)
	IEP (pre)	2000 - 2006	Various wild ungulates (*Ungulata)* and camels (*Camelus)*	3.3% (Overall)	Britch et al. (2013)
	IEP (pre)	2006	Human (*Homo sapiens*)	13%	Labeaud et al. (2008)
	EP	2007	Human (*Homo sapiens*)	4.5%	Ochieng et al. (2015)
			Various wild ungulates (*Ungulata*) and camels (*Camelus)*	31.8%	Britch et al. (2013)

Country	Period	Year	Animal/species	Prevalence	Reference
Kenya	EP	2007	Buffalo (*Syncerus caffer*)	21.4%	Britch et al. (2013)
			Camel *(Camelus dromedarius*)	57%	Britch et al. (2013)
			Eland (*Taurotragus oryx*)	25%	Britch et al. (2013)
			Giraffe (*Giraffa*)	13.33%	Britch et al. (2013)
			Waterbuck (*Kobus ellipsiprymnus*)	33.3%	Britch et al. (2013)
			Gazelle (*Gazella*)	0%	Britch et al. (2013)
			Gerenuk (*Litocranius walleri*)	66.66%	Britch et al. (2013)
			Impala (*Aepyceros melampus*)	100%	Britch et al. (2013)
			Warthog (*Phacochoerus africanus*)	74.4%	Britch et al. (2013)
	IEP(post)	2008-2005	Various wild ungulates (*Ungulata)* and camels (*Camelus)*	5%	Britch et al. (2013)
	IEP(post)	2008-2014	Baboon (*Papio*)	0%	Lwande et al. (2015)
			Black Rhino (*Diceros bicornis*)	28.6%	Lwande et al. (2015)
			Buffalo (*Syncerus caffer*)	17.9%	Lwande et al. (2015)
			Elephant (*Loxodonta africana*)	22.4%	Lwande et al. (2015)
			Eland (*Taurotragus oryx*)	25%	Britch et al. (2013)
			Giraffe (*Giraffa*)	13.33%	Britch et al. (2013)
			Waterbuck (*Kobus ellipsiprymnus*)	33.3%	Britch et al. (2013)
			Gazelle (*Gazella*)	0%	Britch et al. (2013)

Country	Period	Year	Animal/species	Prevalence	Reference
			Gerenuk (*Litocranius walleri*)	66.66%	Britch et al. (2013)
Kenya	IEP(post)	2008-2014	Impala (*Aepyceros melampus*)	100%	Britch et al. (2013)
			Warthog (*Phacochoerus africanus*)	74.4%	Britch et al. (2013)
	IEP(post)	2008-2005	Various wild ungulates (*Ungulata)* and camels (*Camelus)*	5%	Britch et al. (2013)
	IEP(post)	2008-2014	Baboon (*Papio*)	0%	Lwande et al. (2015)
			Black Rhino (*Diceros bicornis*)	28.6%	Lwande et al. (2015)
			Buffalo (*Syncerus caffer*)	17.9%	Lwande et al. (2015)
			Elephant (*Loxodonta africana*)	22.4%	Lwande et al. (2015)
			Giraffe (*Giraffa*)	0%	Lwande et al. (2015)
			Vervet monkey (*Chlorocebus pygerythrus)*	0%	Lwande et al. (2015)
			Warthog (*Phacochoerus africanus*)	8.8%	Lwande et al. (2015)
			Wildebees (*Connochaetes*)	4.8%	Lwande et al. (2015)
			Zebra (*Equus quagga*)	0%	Lwande et al. (2015)
	IEP(post)	2008-2015	Warthog (*Phacochoerus africanus*)	1.96% and 8.8%	Britch et al. (2013)
			Giraffe (*Giraffa*)	0%	Britch et al. (2013)
			Buffalo (*Syncerus caffer*)	2.91% and 19.6%	Britch et al. (2013)

Country	Period	Year	Animal/species	Prevalence	Reference
			Waterbuck (*Kobus ellipsiprymnus*)	16.66%	Britch et al. (2013)
Kenya	IEP(post)	2008-2015	Kudu (*Tragelaphus strepsiceros*)	0%	Britch et al. (2013)
			Eland (*Taurotragus oryx*)	0%	Britch et al. (2013)
			Gerenuk (*Litocranius walleri*)	0%	Britch et al. (2013)
			Eland (*Taurotragus oryx*)	0%	Britch et al. (2013)
			IEP(post)	2010 - 2012	Human (*Homo sapiens*)
			IEP(post)	2013 - 2014	Human (*Homo sapiens*)
Mauritania	EP	2010	Camel *(Camelus dromedarius*)	27.5% and 38.5%	El Mamy et al. (2011, 2014)
Mozambique	IEP	2013 - 2014	Buffalo (*Syncerus caffer*)	30,40%	Moiane et al. (2017)
Nigeria	DNR	1985 - 1989	Human (*Homo sapiens*)	14.8%	Olaleye et al. (1996a)
Nigeria	DNR	1986 - 1989	Pig (*Sus scrofa domesticus*)	0%	Olaleye et al. (1996b)
			Horse (*Equus caballus*)	9.8%	Olaleye et al. (1996b)
			Camel *(Camelus dromedarius*)	3.3%	Olaleye et al. (1996b)
Saudi Arabia	IEP	2008; 2012	Human (*Homo sapiens*)	3.6% and 11%	Al Azraqi et al. (2013); Memish et al. (2015)
Sierra Leone	DNR	2007 - 2014	Human (*Homo sapiens*)	1.8%	O'Hearn et al. (2016)
South Africa	IEP	2015 - 2016	Human (*Homo sapiens*)	8.0% and 9.1%	Msimang et al. (2019)
	IEP	2000 - 2006	Buffalo (*Syncerus caffer*)	21%	Labeaud et al. (2011)
	IEP	2003 - 2004	Buffalo (*Syncerus caffer*)	21.1%	Fagbo et al. (2014)
	IEP	1996 - 2007	Buffalo (*Syncerus caffer*)	0%-27%	Beechler et al. (2015)
	EP	2008	Human (*Homo sapiens*)	15%	Archer et al. (2013)
	EP	2008 - 2012	Buffalo (*Syncerus caffer*)	2.2%	Beechler et al. (2015)
Sudan	IEP (Pre)	1989	Human (*Homo sapiens*)	23%	Watts et al. (1994)

Country	Period	Year	Animal/species	Prevalence	Reference
	EP	2007	Human (*Homo sapiens*)	82%	Hassanain et al. (2010)
	IEP (Post)	2014 - 2015	Camel *(Camelus dromedarius*)	9.6%	Abdallah et al. (2016)
Tanzania	EP	2007 - 2009	Human (*Homo sapiens*)	5.20%	Heinrich et al. (2012)
Yemen	IEP	2013 - 2016	Human (*Homo sapiens*)	65.8%	Al-Aesayi et al. (2019)
Zimbabwe	IEP	1989 - 1995	Warthog (*Phacochoerus africanus*)	0%	Anderson and Rowe (1998)
			Buffalo (*Syncerus caffer*)	6.3%	Anderson and Rowe (1998)
			Sable antelope (*Hippotragus niger*)	0.35%	Anderson and Rowe (1998)
			Eland (*Taurotragus oryx*)	0%	Anderson and Rowe (1998)
			Wildebees (*Connochaetes*)	0%	Anderson and Rowe (1998)
			Giraffe (*Giraffa*)	0%	Anderson and Rowe (1998)
			Waterbuck (*Kobus ellipsiprymnus*)	4.5%	Anderson and Rowe (1998)
			Bushbuck (*Tragelaphus scriptus*)	0%	Anderson and Rowe (1998)
			Nyala (*Tragelaphus angasii*)	0%	Anderson and Rowe (1998)
			White Rhino (*Ceratotherium simum*)	8.3%	Anderson and Rowe (1998)
			Black Rhino (*Diceros bicornis*)	14.5%	Anderson and Rowe (1998)
			Tsessebe (*Damaliscus lunatus*)	0%	Anderson and Rowe (1998)

EP: Epidemic period; IEP: Inter epidemic period; DNR: Disease never reported

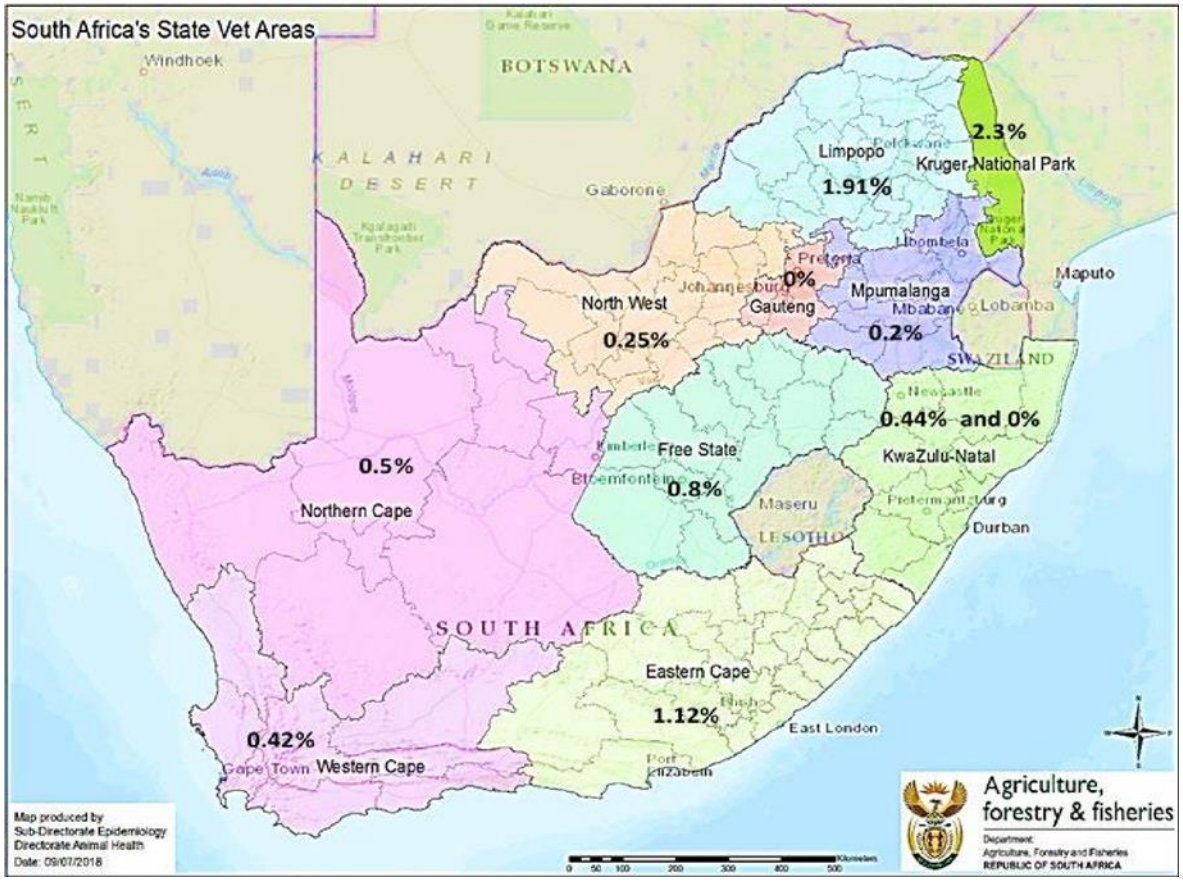

Figure 4.1 Map of South Africa showing the country's 9 provinces and the Kruger National park, with corresponding RVFV antibody seropositivity rates discerned by VNT in this study. True seroprevalence rates in all provinces and the KNP were lower than 0% and are not reflected

4.4 Discussion

Exposure of SA domestic pigs and warthogs to RVFV was demonstrated in this study, albeit at very low seropositivity test levels of 0.68% and 1.87%, respectively. Although cross-reactions with other related viruses in the *Phenuiviridae* family cannot be excluded, warthog and domestic pig positive reactors were confirmed for a subset of samples tested in parallel with a commercial blocking ELISA. Verification of positive results with two tests supports the probability of field exposure of suids to RVFV (Lubisi et al., 2019). It was, however, unfortunately not clear what proportion of domestic pig positive reactors originated from commercial versus subsistence farms. Availability of this information would have been valuable given the marked difference in farming methods between the two sectors. Availability of warthog samples originating from all provinces would have also added value to the current investigation. There were no significant differences ($p > .05$) among the provinces with regard to seropositivity for all the years investigated. South Africa experienced major outbreaks of RVF from 1950–1951, 1973–1975 and 2008–2011 (Métras et al., 2013), with sporadic occurrences reported outside these periods.

In 2008, the disease was reported in MP, LP, GP and NW, whilst in 2009 outbreaks were documented for KZN, EC, MP and NC. All provinces except for KZN reported outbreaks in 2010, and the 2011 outbreaks mainly affected EC, WC and NC (Pienaar & Thompson, 2013). Based on clinical observations and laboratory results, it was estimated that the numbers of dead/infected animals were 103/353 in 2008, 66/210 and 35/53 in 2009, 8877/14342 in 2010 and an undisclosed number/4139 in 2011. Human mortality/infection records were 26/242 during the 2010–2011 outbreaks, whilst 14 and 7 people were infected during the 2008 and 2009 outbreaks, respectively (Archer et al., 2013; Pienaar & Thompson, 2013). Sheep, cattle and goats were the species most affected during the outbreaks, followed by African buffalo (Pienaar & Thompson, 2013), probably because they are farmed and thus passively surveyed. In 2010, however, 9 indigenous and 5 exotic wildlife animals were clinically affected by RVFV, and 5 in 2011 (Pienaar & Thompson, 2013).

In this study, antibodies were detected in EC, KZN and LP in 2008, and EC, LP, FS and NC in 2009. Mpumalanga province, KZN, WC, NW and LP had seropositive pigs in the 2012–2015 period. Neutralizing antibody titres of 1:160 –1:640 were observed for EC (2008 and 2009), LP (2009), FS (2009) and NC (2009). The two positive warthogs were only observed in the 2013– 2016 period, each with titres of 1:160. Comparison of the seroprevalence rates between domestic and wild suids, and cattle, sheep and goats during the above EP and IEP would provide insight into spatio-temporal activities and host preferences of RVFV infected mosquitoes in SA. However, these domestic ruminants are usually vaccinated prior to, during and following outbreaks for preventive and control purposes, and the current lack of DIVA serological assays (Ragan et al., 2018) is a limiting factor

for such studies. Domestic pigs were seemingly only investigated for presence of RVFV antibodies in Egypt during the IEP and Nigeria where the disease was never reported, whilst other studies on suids involved warthogs in Kenya and Zimbabwe where disease is present in both countries (Table 4-2). A number of domestic pigs tested RVFV antibody positive in this study (n = 27) compared to zero in Nigeria, but the percentage of positive reactors in Egypt was higher than in our study (Olaleye et al., 1996b; Youssef, 2009). Different husbandry and farming methods, and laboratory tests employed, likely explain the disparities observed with the latter population. For warthogs, seroprevalence rates observed in this study were lower than those observed in Kenya during the IEP, with no positive reactors detected among warthogs from Zimbabwe during similar periods (Table 4.2). Positive samples were only reported in Kenya during the EP. Availability of diagnostic test parameters of the assays used to discern seroprevalence rates in RVFV permissive mammalian hosts other than amplifying domestic ruminants in endemic countries would have allowed for statistical and informative comparison of our results with those cited in Table 4.2. This limitation was a major finding by Clark et al. (2018), who reported that the majority of the 126 RVF seroprevalence articles they reviewed did not account for study design bias or test diagnostic sensitivity and specificity. Based on sera availability, quantity and quality, the sampling method used in the study resulted in some years, provinces, state veterinarian areas and farms being excluded, under or over represented, thus creating selection bias (Šimundić, 2013). Similar challenges were observed with other studies, especially those involving wildlife species (Table 4.2). The test seroprevalence rates recovered in this study may thus not necessarily represent true population values in suids but nonetheless provide evidence of RVFV exposure in this understudied family (Table 4.1), similar to the proof of exposure in less distinctive mammalian hosts afforded by various workers in Southern Africa and other countries (Table 4.2). As true prevalence rates discerned from mathematical correction of VNT results for domestic pigs and warthogs for all years sampled were less than zero (Table 4.1), we opted to report test prevalence rates. This is because the diagnostic sensitivity and specificity, which were integral to calculation of the true prevalence rates, were determined by comparing VNT performance to that of a competition multi-species ELISA. Although the ELISA is presumed to work in all species, it has not specifically been evaluated in suid populations, making it an imperfect reference standard (Lewis & Torgerson, 2012; Lubisi et al., 2019).

Limited data make proper validation of serological tests for use in suids a serious challenge that undoubtedly affects determination of true and unbiased test performance characteristics, and consequently the accuracy of calculations of the disease's true seroprevalence in suid populations.

Domestic pigs have a real time spatio-temporal existence with mammalian hosts, vectors and RVFV. This is because they are integral to Africa's mixed species farming systems, come into contact with humans, are bitten by mosquitoes, and scavenge and eat dead animals including aborted foetuses (Kamgang et al., 2012; Mahande et al., 2007; Thomas et al., 2013; Wabacha et al., 2004). Since ruminants and humans can acquire RVFV infection through mosquito bites, and humans also get infected *via* contact with infected carcasses and body fluids when manipulating them, it is probable that pigs can be infected in the same manner. Infection in this species is, however, mostly subclinical and laboratory detection of viral antibodies or antigen may be the only feasible method of ascertaining exposure and infection.

The seemingly low RVFV infection rates seen in suids could, however, be due to: i) pigs requiring very high titres of virus compared to domestic ruminants for establishment of infection, which may not be present in the viral source (Scott, 1963); ii) preference of *Aedes* sp., *Anopheles* sp. and *Culex* sp., to feed on humans followed by cattle and other domestic animals, with feeding on pigs being an indication of plasticity when the preferred hosts are scarce (Mahande et al., 2007; Mwandawiro et al., 2000; Ponlawat & Harrington, 2005; Takken & Verhulst, 2013); iii) bites by uninfected mosquitoes; iv) deposition of the virus in pig subcutaneous fat by mosquitoes resulting in unsuccessful infection (Svoboda & Drabek, 2007) and v) low antibody titres which may be undetectable by the VNT used (Lubisi et al., 2019; Chapter 3). Apart from their anatomy and the above listed factors, determination of the most effective RVFV infection route for pigs and warthogs could explain the seroprevalence rates observed under different field conditions, and the generally lower rates compared to other atypical RVFV host species (Fig 4.1; Table 4.2).

Globalization and global warming are nonetheless the biggest drivers of disease emergence, characterised by outbreaks of previously unknown infections or changes in the epidemiology of known diseases (Dhama et al., 2013; Sutherst, 2004). Diseases caused by *Bunyaviruses* are emerging and require structured and constant monitoring. For example: i) Schmallenberg was unknown and recently identified for the first time in Europe (Hoffmann et al., 2012); ii) Akabane clinically crossed the species barrier to affect pigs in Asia (Huang et al., 2003) and iii) RVF increased incidence rates and clinically affected a number of wildlife animals in Africa (Pienaar & Thompson, 2013) and emerged in 2000 in Saudi Arabia and Yemen which were both previously uninfected (Ahmad, 2000; Himeidan et al., 2014).

This is the first serological surveillance of RVF in South African domestic pigs and current findings suggest that pigs and warthogs do not play a significant role in the epidemiology of RVF in the country. Dearth of information with regard to RVF epidemiology in endemic countries, however,

remains, and continued research on factors enabling field RVFV maintenance and spread is warranted. This should include surveillance programmes involving mosquito vectors and known vertebrate and various potential mammalian reservoir hosts to enable timeous identification of changes in the epidemiology of the disease, performance of risk analysis and institution of mitigation measures when necessary.

CHAPTER 5

GENERAL DISCUSSION

Many strides have been made in determining and understanding RVFV, its vertebrate and invertebrate hosts, pathogenesis and clinical and post mortal signs, epidemiology, and diagnostic test and vaccine development since 1931 when the disease was first reported, which enabled devising of the current control measures. However, knowledge gaps which require scientific answers in order to advance RVF control and perhaps eradication, still exist. Some of these unanswered questions according to Bird and McElroy (2016) include:

1. RVFV pathogenesis

a) What genetic factors and co-morbidities contribute to severe disease outcomes in humans?

b) Why is human maternal to foetal transmission of RVFV so rare?

c) What underlies the pathogenesis of RVF retinitis and delayed-onset encephalitis?

d) Can targeted antiviral therapeutics that can cross the blood-brain-barrier be effective in treating RVF neurologic disease?

e) What is the importance of cell-mediated immunity against RVFV?

2. Wildlife and risk factors

a). What is the importance (if any) of cryptic small mammal hosts or other wildlife in “ancestral homelands” and endemic areas?

b). Are common European and North American wildlife and non-traditional livestock susceptible to infection and disease and can mosquito vectors in these areas trans-ovarially transmit RVFV?

c). What is the virus load in raw milk and meat products from infected animals?

3. Control and eradication strategies

a). Can one-health vaccination approaches for livestock actually contain virus spread and reduce human infections?

b). Can we develop integrated comprehensive eradication plans for successful control of RVFV following introduction into non-endemic areas?

In an attempt to answer the question related to risk factors and importance of alternative/overlooked hosts in endemic areas, we investigated the potential role of domestic pigs in the epidemiology of RVF by serologically determining their natural exposure and that of warthogs to RVFV, and experimentally investigating the effect of virus infection on pregnant sows, suckling piglets and weaners.

Our study has shown that the virus neutralization test is the best serological assay for testing porcine sera and plasma for RVF antibodies in the absence of well validated tests for use in this species. It has also demonstrated that even though seroprevalence rates for domestic pigs and warthogs were low at 0.68% and 1.87% respectively, the species were exposed to RVFV, probably *via* mosquito bites, and seroconverted. The results showed lower test prevalence rates than those obtained from domestic pigs in Egypt (Youssef, 2009) and warthogs in Kenya (Britch et al., 2013; Evans et al., 2008; Lwande et al., 2015), but the effects of sampling and assays utilized in the different studies cannot be ruled out. Successful experimental infections were achieved, characterised by fever in some animals, seroconversion and demonstration of viral RNA in organs, blood and secretions for at least 28 days, and by abortion and reproductive disorders. Also notable in our study was seroconversion and demonstration of viral RNA in tissues, blood and secretions of in-contact control sheep, sows, weaners and suckling piglets.

Despite the fact that our findings were obtained at experimental levels, they can be considered for incorporation in the current RVF control measures, irrespective of the fact that the levels and duration of viraemia in the pig blood, secretions and excretions have not been determined yet. Since we have proven that pigs are exposed to RVFV and seroconvert, probably due to mosquito bites, their sera may be tested for RVFV antibodies as well during targeted pig disease sero-surveillance exercises, especially if located near rivers, swamps and dams. This would be a way of monitoring vector activity, thus providing additional early warning for RVF incursion. Despite this potential use of sera from pigs living in environments which are conducive for proliferation of mosquitoes, clinically susceptible ruminants should be used as primary sentinels since they readily seroconvert following bites by infected mosquitoes, while the fat in the pig's skin may interfere with successful infection and seroconversion (Groswasser et al., 1997; Michaels & Poole, 1970; Poland et al., 1997).

Areas targeted for mosquito vector control near RVF susceptible species can also be extended to pig habitats, which in many developing countries are still informal and conducive to mosquito breeding (Sithole et al., 2020; Temu et al., 2012; Wabacha et al., 2004).

A ban on movement of susceptible animals is imposed to prevent transfer of potentially viraemic animals which may serve as sources of virus for vectors and initiate outbreaks in clean areas. Even though this study has proven pigs to be permissive to RVFV infection, and viral RNA detected in their blood, excretions (oral-nasal and faecal swab pools) and tissues, and virus isolated from their organs, a ban on pig movement along with that of ruminants during outbreaks cannot be recommended in the absence of robust laboratory and field based scientific evidence of their virus

shedding, and ability to successfully infect vector mosquitoes. While experimental studies cannot always be extrapolated to the field, our findings have provided a case that provides merit for further research on the possible role of this species in RVFV transmission to vector mosquitoes and other susceptible species, especially during disease outbreaks.

Separating pigs from domestic ruminants during outbreaks could be considered, for prevention of possible transfer of virus from infected ruminants to pigs, for food safety reasons, especially when the pigs are due for slaughtering and consumption during outbreaks. This is informed by the observation that uninfected lactating sheep and sows, weaners and suckling pigs, seroconverted to, and tested RVFV RNA positive on PCR, proving that horizontal transmission took place when they were housed with infected lambs, sows and neonatal piglets.

In addition, precautions similar to those taken for ruminants when handling aborted foetuses and dead animals, and burying them during outbreaks, should be applied for the pigs as well. This is because abortion was observed and the aborted foetuses were positive for RVFV RNA and antigen on real time RT-PCR and IHC, and the virus was demonstrated in liver samples of three of the foetuses by EM. Virus antigen was also demonstrated in the livers, spleens and kidneys of piglets born from infected sows.

Despite reported seroconversion, pigs were never included among RVFV susceptible animals nor regarded as important in RVF epidemiology. Our findings have nonetheless shown that under experimental conditions, pigs can be viraemic and potentially infective for at least 28 days post infection. This could warrant their inclusion in ring vaccinations to prevent further spread of the virus during outbreaks should the outcome of future field studies so dictate, and their response to vaccination with currently available and licensed vaccines be positive. Cost benefit analysis by responsible authorities should also favour such interventions. Since it has been proven that they require high viral doses for meaningful and successful infection, and are generally clinically less susceptible, pigs and pregnant sows could be vaccinated with classical live attenuated formulations such as the Smithburn strain based ones, while the nonabortogenic vaccines are reserved for ruminants.

There were shortcomings observed in all three parts of our study. For the sero-prevalence determination, the laborious virus neutralization test (VNT) was used instead of the rapid commercial blocking ELISA due to resource constraints, and this exposed the reading of results of weak positive or suspect samples to human subjectivity. Nonetheless this allowed for the VNT to

be evaluated against the commercial ELISA and it was revealed that the ELISA was less sensitive with regard to IgM antibody detection (Lubisi et al., 2019; Chapter 3). This highlighted the need to validate ELISAs using samples from the intended species, irrespective of their claim of suitability for use in all species due to their antigen blocking nature. Warthog sample numbers were far lower than those of domestic pig, and were also geographically constrained, represented by just three provinces (KZN, MP, LP). Also, information regarding the farming systems from which the domestic pig samples were obtained was lacking. These two limitations frustrated efforts to obtain possibly important epidemiological information on differences in prevalence among provinces and between commercial and subsistence farming systems with regard to warthogs and domestic pigs respectively.

Drawbacks related to the experimental infections included the fact that a pig RVF infection model does not exist and infection was done via the jugular vein, which does not mimic routes of natural infection under field conditions. Five experiments were run concurrently and challenges were experienced with the time taken to perform procedures, collection of too many specimens, treatment and preparation of samples for removal from the BSL3 facility, transportation to the laboratory where they were going to be tested, availability of suitable storage spaces, and other necessary resources. As a result, plasma proteins C-reactive protein (CRP), serum amyloid A (SAA) and haptoglobin, which constitute acute phase proteins (APP) produced by the liver as part of a host systemic response to infection, inflammation or trauma, in a pathophysiological process called acute-phase response (APR), were not analysed. The maximum serum concentration of APPs is normally reached within 24 to 48 h after stimulation and a decline is seen afterwards, with resolution observed within 4 to 7 days after the initial stimulus (Jain et al., 2011).

Samples were processed after a year of storage, tissues were stored at -20^0 Celsius and underwent a few freeze-thaw cycles prior to processing, and blood was haemolysed. This undoubtedly affected the quality of the samples and contributed to the rejected samples for VNT, and number of negative results obtained, especially with PCR and virus isolation due to RNA degradation. The delays in sample processing and poor storage not only affected analysis of APPs and diagnostic PCR results, but affected determination of viral loads from different samples throughout the collection period by virus quantification using real time RT-PCR and micro or plaque titration methods.

Limited financial resources resulted in inability to test all samples for RNA presence or put them all up for virus isolation. All sera or plasma from experimental animals (sheep and pigs) were tested for RVFV antibodies using the commercial IDVet blocking ELISA but only blood and swabs from

animals which tested antibody positive were analysed for viral RNA presence on real-time RT-PCR. It is highly likely that a considerable number of samples collected early during infection and with good prospects of testing PCR positive, were omitted from the PCR test panel. This is based on the fact that the IDVet RVFV antibody ELISA kit on whose results sample selection decisions were made, was proven to be less sensitive than the VNT in RVFV IgM and to a lesser extent, IgG antibody detection (Lubisi et al., 2019; Chapter 3).

In addition to testing only a few selected blood and swab samples and possibly excluding a number of positive ones, the processed swabs for PCR testing were pooled, similar to organ samples put up for virus isolation. Pooling of samples is known to reduce success of obtaining positive results due the dilution effect of negative samples on the positive ones. This could have been the case for all sample pools analysed for viral RNA presence and virus isolation, with negative outcomes. Attempts to isolate virus from the swab pools were not made.

Future studies must focus on full genome analysis of the pig passaged RVFV to clarify presence of virus variants of different lineages and determine any possible recombination and reassortment events, especially where two different strains were used in a single stable. Such an undertaking will assist in determining the pig's potential of being a mixing vessel for RVFV, as it is for influenza viruses (Scholtissek, 1990). Future studies must also address the above mentioned shortcomings of the current study, and focus on elucidating the pathogenesis of RVFV in porcine hosts, host immune response to RVF infection, and pig-vector interactions, given the findings of this study. Advantage should be taken of the fact that pigs are known to be anatomically, immunologically and physiologically similar to humans and are acceptable subjects in human biomedical research (Swindle & Smith, 1998). Following establishment that pigs can be successfully infected with RVFV, Bird and McElroy's (2016) unanswered pathogenesis related question on the genetic factors and comorbidities that contribute to severe disease outcomes in humans could be first investigated in pigs through co-infecting them with RVFV and another zoonotic virus such as Hepatitis E virus, and observing genetic responses.

Social sciences could account for why vertical transmission of RVFV among humans is seemingly rare, with potential reasons such as under representation of pregnant women in careers that involve constant exposure to and handling of susceptible livestock given, and under reporting and inadequate investigation of possible losses of pregnancy among women in poor rural farming communities (Archer et al., 2013; Baudin et al., 2016). Even though the human placenta is different from that of the sow (Burton & Juaniaux, 2018; Roberts et al., 2016), life scientists could utilise

pregnant sows to investigate possible determinants of human mother to child transmission of RVF *in-utero*, given the fact that only 1 out of 7 confirmed RVFV infected pregnant sows aborted in our study. Retinitis and delayed-onset encephalitis were not observed in our experiments, and cellmediated immunity against RVFV and effectiveness of targeted antiviral therapeutics that can cross the blood-brain-barrier in treating RVF neurologic disease were not investigated, but the pig model could be used in such endeavours. This is due to their susceptibility to infection and their anatomical and physiological similarities, especially the holangiotic retinal vasculature, lack of tapetum, cone photoreceptors in the outer retina, and comparable scleral thickness (Middleton, 2010)

On unanswered questions related to wildlife and risk factors (Bird & McElroy, 2016), seroconversion of warthogs to RVFV has been demonstrated in the current and previous studies (Britch et al., 2013; Evans et al., 2008; Lubisi et al., 2020; Lwande et al., 2015), thus potential involvement of this species in the epidemiology of RVF was highlighted. Future studies may involve experimental infection of warthogs with RVFV to determine possible viral amplification, pathogenesis, shedding and duration of shedding, and possible carrier status. The potential importance of atypical mammalian hosts in endemic areas has been addressed using the domestic pig in this study, and future workers can select other species to investigate. Determination of viral loads in raw milk and meat products of infected pigs would definitely be a positive contribution to the body of knowledge.

Conclusion

Emerging zoonotic diseases such as RVF have potentially devastating human and animal health, and economic impact. Changes in human behaviour, farming and trading practices, climatic and environmental conditions, vector distribution and the genes of pathogens contribute to disease emergence through facilitation of pathogen transfer between animals and species barrier crossing (Parrish et al., 2008). Once disease agents switch hosts, factors determining pathogenicity and other mechanisms that establish them in populations and make them transmissible in the new species are poorly understood (Longdon et al., 2014). Some of the pathogens may result in clinical or sub-clinical infections in non-reservoir species, as may potentially be the case with RVF in pigs, since successful experimental infection of this species with RVFV, sow abortion, birth of RVF antibody and virus positive offspring, and potential shedding of virus in the oronasal secretions of the pigs for 28 DPI was proven in this study. Given the fact that drivers of disease emergence play different roles in occurrence, spread and maintenance of zoonotic diseases as attested by transmission of RVFV in mock and uninoculated pigs housed with infected ones through an unknown mechanism, a onehealth approach in all aspects of outbreak investigation, surveillance

and monitoring in known RVF hosts should be adopted, and where possible, involve epidemiologically linked, known hosts and atypical species such as pigs and other ungulates in close contact. Such information can be used in system dynamics modelling for public and veterinary health for better understanding of the underlying forces of RVF transmission and improved prevention and control of the disease, thus mitigating its negative impact on human and animal health, and the economy (Grace et al., 2017; Homer & Hirsch, 2006).

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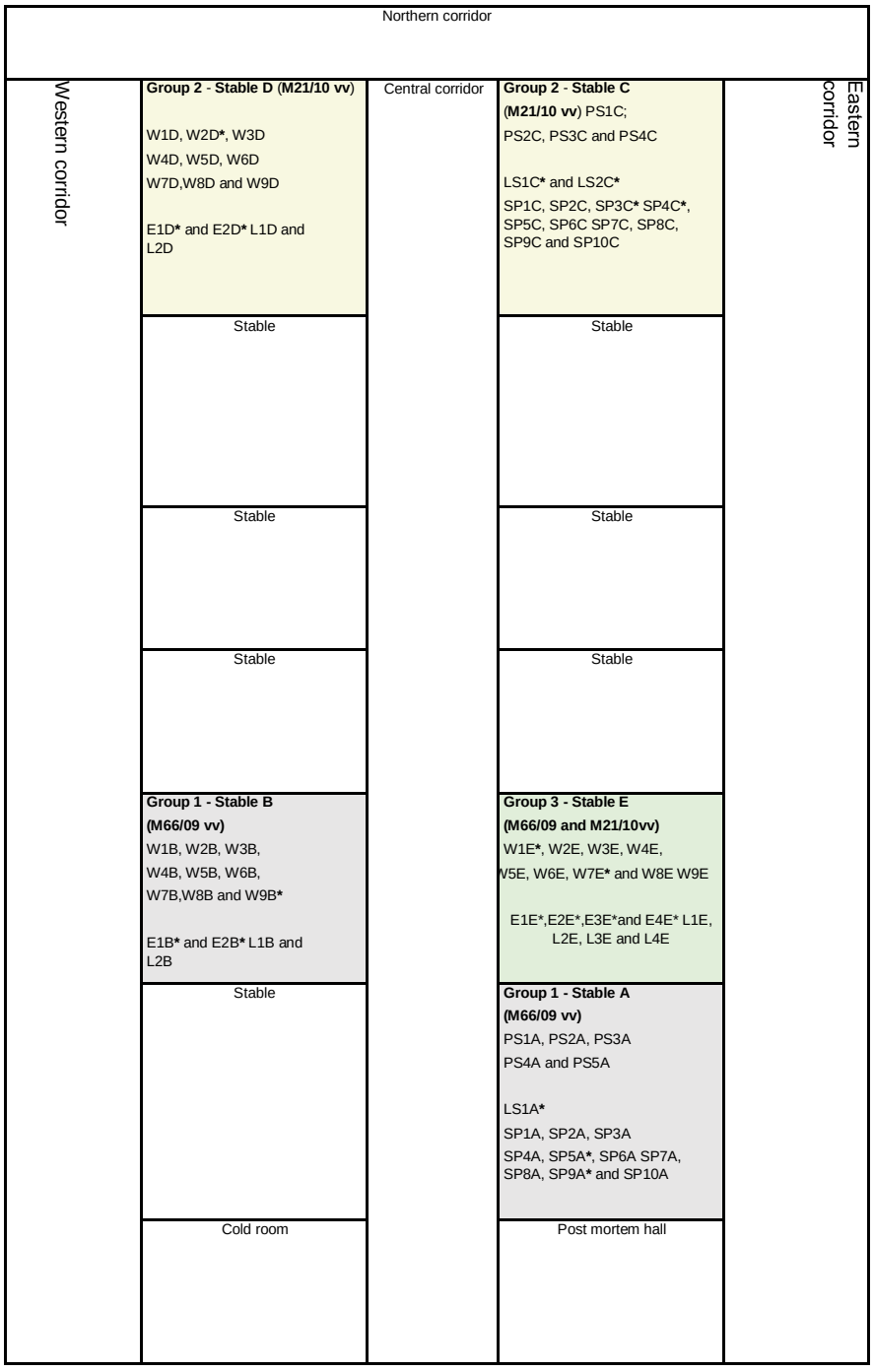
APPENDICES

Appendix 2.1 Schematic diagram of the stables in which the experimental animals were housed. Movement was from stables A to B, followed by C to D, then E, using the eastern, western, and northern corridors. The central corridor was used to take dead animals to the post mortem hall or cold room. Proper biosafety and biosecurity procedures were followed and PPE was used for personnel safety and avoidance of cross contamination. Virus 1 (M66/09 variant) and virus 2 (M21/10 variant) were used to inoculate animals in groups 1 (stables A and B) and 2 (stables C and D) respectively, whereas group 3 animals (stable E) were either inoculated with virus 1 (W2E and W5E) or virus 2 (W4E and W6E) or with a mixture of the two viruses (L1E, L2E, L3E and L4E, and W3E, W8E and W9E). The control piglets and weaners were mock inoculated with TC medium and the ewes and lactating sows received no treatment. The stable codes constituted the animal identity suffixes



Appendix 2.2 Laboratory test results of group 1 animals. Only blood and swab pools of animals that demonstrated antibody presence on ELISA were tested on real time RT-PCR (newborn piglets were not swabbed). For blood, swab pools and sera, a negative result represents a collection of negative results of all the samples tested for the particular animal

Animal	Real time RT-PCR			Conventional RT-PCR	ELISA
	Organ pool (DPI)	Blood (DPI)	Oronasorectal swab pool (DPI)	Tissue culture material	Serum (DPI)
PS1A	-	-	+ (3; 4)	+	+ (21; 28; 61)
PS2A	-	-	+ (2; 4)	+	Susp (14); + (21; 28)
PS3A	-	-	-	+	Susp (21); + (28)
PS4A	-	-	-	-	+ (14; 21)
PS5A	-	-	-	+	+ (13; 14)
PS2A-P1	+ (23)	-	N/A	NT	NT
PS2A-P2	-	-	N/A	-	NT
PS2A-P3	+ (23)	-	N/A	-	Susp (23)
PS2A-P4	+ (23)		N/A	-	Susp (23)
PS2A-P5	-	-	N/A	-	Susp (23)
PS2A-P6	+ (23)	-	N/A	-	-
PS2A-P7	-	-	N/A	-	Susp (23)
PS2A-P8	-	-	N/A	+	-
PS2A-P9	-	-	N/A	-	Susp (23)
PS2A-P10	+ (23)	-	N/A	+	-
PS2A-P11	+ (23)	-	N/A	+	-
PS2A-P12	+ (23)	-	N/A	+	-
PS2A-P13	-	-	N/A	-	Susp (23)
PS2A-P14		-	N/A	-	-
PS2A-P15	-	-	N/A	+	NT
PS3A-P1	-	-	N/A	-	Susp (28)
PS3A-P2	N/A	-	N/A	NT	+ (28)
PS3A-P3	-	-	N/A	-	+ (28)

Animal	Real time RT-PCR			Conventional RT-PCR	ELISA
	Organ pool (DPI)	Blood (DPI)	Oronasorectal swab pool (DPI)	Tissue culture material	Serum (DPI)
PS3A-P4	-	-	N/A	+	+ (28)
PS3A-P5	+ (28)	-	N/A	+	+ (28)
PS3A-P6	-	-	N/A	-	+ (28)
PS3A-P7	+ (28)	+ (28)	N/A	+	+ (28)
PS3A-P8	-	-	N/A	NT	+ (28)
PS3A-P9	-	-	N/A	-	+ (28)
PS3A-P10	+ (28)	-	N/A	NT	+ (28)
PS3A-P11	+ (28)	-	N/A	+	Susp (28)
PS3A-P12	-	-	N/A	NT	+ (28)
PS3A-P13	-	-	N/A	-	NT
PS3A-P14	-	-	N/A	-	NT
PS4A-P1	+ (32)	-	N/A	-	+ (32)
PS4A-P2	+ (32)	-	N/A	-	+ (32)
PS4A-P3	-	-	N/A	-	+ (32)
PS4A-P4	-	-	N/A	-	+ (32)
PS4A-P5	-	-	N/A	-	+ (32)
PS4A-P6	+ (32)	-	N/A	-	+ (32)
PS4A-P7	-	-	N/A	-	+ (32)
PS5A-AF1 to AF12	+ AF1 (14) + AF5 (14)	NT	N/A	+ AF5; AF6; AF9	NT
LS1A	NT	NT	+ (5)	NT	+(14)
SP1A	NT	NT	NT	NT	-
SP2A	NT	NT	NT	NT	-
SP3A	- (4)	NT	NT	NT	-
SP4A	+ (4)	NT	NT	NT	-
SP5A*	- (21)	NT	NT	NT	-
SP6A	- (5)	NT	NT	NT	-
SP7A	+ (5)	NT	NT	NT	-
SP8A	- (15)	NT	NT	NT	-
SP9A*	+ (21)	NT	NT	NT	-
SP10A	- (12)	+ (3;4)	+ (4)	NT	+ (14)
E1B	- (29)	NT	NT	NT	-
E2B	- (29)	NT	NT	NT	-
L1B	- (29)	-	+ (1 ; 21)	NT	+ (29)

Animal	Real time RT-PCR			Conventional RT-PCR	ELISA
	Organ pool (DPI)	Blood (DPI)	Oronasorectal swab pool (DPI)	Tissue culture material	Serum (DPI)
L2B	+ (29)	-	-	NT	+ (29)
W1B	- (2)	NT	NT	NT	-
W2B	- (4)	NT	NT	NT	-
W3B	- (6)	NT	NT	NT	-
W4B	- (15)	NT	NT	NT	-
W5B	+ (21)	-	+ (3;4)	NT	Susp (14;21)
W6B	- (29)	-	-	NT	+ (21;28;30)
W7B	- (61)	-	+ (21;28)	NT	+ (30)
W8B	- (61)	NT	NT	NT	-
W9B*	- (61)	-	NT	NT	Susp (21)

PS: Pregnant sow; LS: Lactating sow; SP: Suckling piglet; W: Weaner; L: Lamb; E: Ewe; vv: Virus variant; *: Negative control; *: Negative control; NT: Not Tested; N/A: Not applicable; +: Positive; -: Negative; Susp: Suspect; DPI: Days Post Infection; DEPI: Day Euthanised Post Infection or Use

Appendix 2.3 Laboratory test results of group 2 animals. Only blood and oronasorectal swab pools of animals that demonstrated antibody presence on ELISA were tested on real time RT-PCR (newborn piglets were not swabbed). For blood, oronasorectal swab pools and sera, a negative result represents a collection of negative results of all the samples tested for the particular animal

Animal	Real Time RT-PCR			Conventional RT-PCR	ELISA
	Organ pool (DPI)	Blood (DPI)	Oronasorectal swab pool (DPI)	Tissue culture material	Serum (DPI)
PS1C	-	N/A	N/A	+	-
PS2C	-	-	-	+	Susp (4); + (14; 21; 28; 61)
PS3C	-	-	+ (21)	-	+ (4; 14; 21; 27)
PS4C	-	N/A	N/A	-	-
PS1C-P1	-	-	N/A	-	-
PS1C-P2	+ (27)	-	N/A	+	-
PS1C-P3	+ (27)	+ (27)	N/A	NT	-
PS1C-P4	+ (27)	-	N/A	+	-
PS1C-P5	- (27)	-	N/A	-	-
PS1C-P6	- (27)	-	N/A	-	-
PS1C-P7	+ (27)	-	N/A	NT	-
PS1C-P8	+ (27)	-	N/A	+	-

Animal	Real time RT-PCR			Conventional RT-PCR	ELISA
	Organ pool (DPI)	Blood (DPI)	Oronasorectal swab pool (DPI)	Tissue culture material	Serum (DPI)
PS1C-P9	- (27)	-	N/A	+	-
PS1C-P10	+ (27)	-	N/A	-	-
PS1C-P11	+ (27)	-	N/A	-	-
PS1C-P12	+ (27)	NT	N/A	+	-
PS1C-P13	- (27)	NT	N/A	NT	NT
PS1C-P14	+ (27)	NT	N/A	+	NT
PS1C-P15	+ (27)	NT	N/A	NT	NT
PS1C-P16	-	NT	N/A	NT	NT
PS1C-P17	+ (27)	NT	N/A	-	NT
PS2C-P1	-	-	N/A	-	NT
PS2C-P2	-	-	N/A	-	NT
PS2C-P3	-	-	N/A	+	-
PS2C-P4	+ (44)	-	N/A	+	+ (44)
PS2C-P5	-	-	N/A	-	+ (44)
PS2C-P6	+ (44)	-	N/A	+	+ (44)
PS2C-P7	-	-	N/A	+	+ (44)
PS2C-P8	+ (44)	-	N/A	+	+ (44)
PS2C-P9	-	-	N/A	-	+ (44)
PS2C-P10	-	-	N/A	NT	NT
PS2C-P11	+ (44)	-	N/A	NT	NT
PS3C-P1	-	-	N/A	NT	+ (22)
PS3C-P2	+ (22)	-	N/A	-	Susp (22)
PS3C-P3	+ (22)	-	N/A	-	+ (22)
PS3C-P4	-	-	N/A	NT	Susp (22)
PS3C-P5	+ (22)	-	N/A	NT	-
PS3C-P6	+ (22)	-	N/A	-	+ (22)
PS3C-P7	+ (22)	-	N/A	-	+ (22)
PS3C-P8	+ (22)	-	N/A	+	+ (22)
PS3C-P9	-	-	N/A	NT	Susp (22)
PS3C-P10	-	-	N/A	NT	+ (22)
PS3C-P11	+ (22)	-	N/A	-	Susp (22)
PS3C-P12	NT	NT	N/A	+	NT
PS3C-P15	NT	NT	N/A	-	NT
PS4C-P1	-	NT	N/A	NT	-
PS4C-SB2	NT	NT	N/A	-	NT

Animal	Real Time RT-PCR			Conventional RT-PCR	ELISA
	Organ pool (DPI)	Blood (DPI)	Oronasorectal swab pool (DPI)	Tissue culture material	Serum (DPI)
PS4C-SB3	-	NT	N/A	-	NT
PS4C-P3	NT	NT	N/A		-
PS4C-P4	-	NT	N/A	NT	-
PS4C-P5	+ (32)	NT	N/A	+	-
PS4C-P6	-	NT	N/A	-	-
PS4C-P7	-	NT	N/A	+	-
PS4C-P8	-	NT	N/A	-	-
LS1C	+ (22)	NT	NT	NT	-
LS2C	+ (22)	NT	NT	NT	-
SP1C	NT	NT	NT	NT	-
SP2C	NT	NT	NT	NT	-
SP3C*	- (22)	NT	NT	NT	-
SP4C*	- (22)	NT	NT	NT	-
SP5C	+ (4)	NT	NT	NT	-
SP6C	+ (4)	NT	NT	NT	-
SP7C	NT	NT	NT	NT	-
SP8C	+ (6)	NT	NT	NT	-
SP9C	NT	NT	+ (6;7)	NT	Susp (1); + (14)
SP10C	- (4)	NT	NT	NT	-
E1D	+ (3)	NT	NT	NT	-
E2D	+ (3)	NT	NT	NT	-
L1D	+ (3)	NT	NT	NT	-
L2D	+ (3)	+ (2)	-	NT	-
W1D	NT	NT	NT	NT	+ (3)
W2D*	- (62)	-	+ (5;7;14)	NT	+ (14;21); Susp (28)
W3D	- (5)	NT	NT	NT	-
W4D	+ (14)	-	+ (5;6;7)	NT	+ (7;14)
W5D	- (22)	-	+ (5;6;14)	NT	+ (14;21)
W6D	- (30)	+ (1)	+ (4;6)	NT	+ (21;28)
W7D	- (62)	-	-	NT	+ (21;28)
W8D	- (3)	NT	NT	NT	-
W9D	- (62)	+ (21)	+ (5;6)	NT	Susp (14); Pos (21;28)

PS: Pregnant sow; LS: Lactating sow; SP: Suckling piglet; W: Weaner; L: Lamb; E: Ewe; vv: Virus variant; *: Negative control; *: Negative control; NT: Not Tested; N/A: Not applicable; +: Positive; -: Negative; Susp: Suspect; DPI: Days Post Infection; DEPI: Day Euthanised Post Infection or Use

Appendix 2.4 Real time RT-PCR and blocking ELISA results of group 3 animals. For blood, oronasorectal swab pools and sera, a negative result represents a collection of negative results of all the samples tested for the particular animal

Animal	Real time RT-PCR			ELISA
	Organ pool (DEPI)	Blood (DPI)	Oronasorectal swab pool (DPI)	Serum (DPI)
L1E♥	- (29)	+ (29)	+ (1;3;5;7;14)	Susp (5); + (21)
L2E♥	- (29)	-	+ (3)	Susp (5); + (21)
L3E♥	- (29)	-	NT	+ (29)
L4E♥	- (29)	-	NT	+ (5;29)
E1E	- (29)	NT	NT	-
E2E	- (29)	NT	NT	-
E3E	- (29)	NT	NT	-
E4E	- (29)	NT	NT	-
W1E*	+ (29)	NT	NT	-
W2E▪	- (29)	+ (5)	-	+ (14;30)
W3E♥	+ (29)	NT	NT	-
W4E▲	- (29)	-	+ (7)	+ (14;30)
W5E▪	- (29)	+ (1)	-	Susp (30)
W6E▲	- (29)	NT	NT	-
W7E*	- (29)	-	-	Susp (14); + (30)
W8E♥	- (29)	-	-	+ (30)
W9E♥	- (29)	-	-	-

L: lamb; E: Ewe; W: Weaner; *: Negative control; NT: Not Tested; +: Positive; -: Negative; Susp: Suspect; DPI: Days Post Infection; ♥: Inoculated with virus mixture; ▪: Infected with virus 1; ▲: Inoculated with virus 2; DEPI: Day Euthanised Post Infection or Use

Appendix 2.5 Comparison of results of RVFV infectivity experiments in weaners conducted in this study and that of Clarke et al., 2021

Analyte	Sample	Proportion positive (%)		*P* - value
		This study	Clarke et al., 2021	
Antibody	Serum/Plasma	53.63 (n = 19)	100 (n = 6)	0.04 (95%CI: -2.6% to 67%)
Virus (isolation)	Organs	NT	0 (n = 6)	N/A
	Rectal swabs	NT	0 (n = 6)	N/A
	Blood/Serum	NT	50 (n = 6)	N/A
	Oronasal swabs	NT	33.33 (n = 6)	N/A
RNA	Blood/Serum	20 (n = 10)	0 (n = 6)	0.256 (95%CI: -21.6% to 51%)
	Oronasal/oronasorectal swabs	60 (n = 10)	0 (n = 6)	0.02 (95%CI: -11.5% to 83%)
	Organs	11.11 (n = 18)	NT	N/A
RNA	TC material - Serum	NT	50 (n = 6)	N/A
	TC material - oronasal swabs	NT	33.33 (n = 6)	N/A

TC: Tissue culture; NT: Not tested; N/A: Not applicable