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AN INVESTIGATION OF AN OUTBREAK OF RIFT VALLEY FEVER ON A CATTLE FARM IN BELA-BELA, SOUTH AFRICA IN 2008

by

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Submitted in partial fulfilment of the requirements for the degree of
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November, 2011

In memory of my beloved Mum

DECLARATION

I declare that this dissertation hereby submitted to the University of Pretoria for the degree of MSc (Veterinary Tropical Diseases) has not been previously submitted by me or anyone for the degree at this or any other University, that it is my own work in design and in execution, and that all material contained therein has been duly cited.

Signature

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(Lourenço Paulo Mapaco)

Pretoria, ___/___/2011

This dissertation forms part of the requirements for a web-based MSc degree research project in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. These projects carry a weight of approximately 100 credits, and are therefore smaller than projects required for a research-based MSc degree with a weight of 240 credits. It would be appreciated if reviewers could evaluate the dissertation in that context.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following persons and institutions, without whose assistance this work could not have been done:

My supervisor, Prof. Estelle H. Venter, for her guidance, criticism, ideas and continuous correction of the manuscript.

My co-supervisors, Prof. J.T. Paweska and Prof. J.A.W. Coetzer for their objective suggestions and assistance.

My local advisors, Prof. L. Neves and Dr. J. Fafetine for their unconditional assistance.

Dr. P. Jansen van Vuren, Dr. Antoinette Grobbelaar, Dr. Jacqueline Weyer, Ms. Pat Leman of the National Institute for Communicable Diseases (NICD) of the National Health Laboratory Service (NHLS), Ms. Karen Ebersohn of the Department of Veterinary Tropical Diseases for their support and assistance in the laboratory, and Ms. Rina Serfontein and Fransie Lottering for administrative arrangements.

My family for providing the social environment.

The Veterinary Services of Limpopo Province for sample collection, and Mr. Botes and Mr. Dinkelman for the animals to be bled.

The Department of Veterinary Tropical Diseases, University of Pretoria for sponsoring the project, and the Instituto de Investigação Agraria de Moçambique for additional support.

ABBREVIATIONS

ABTS	Azino di-ethyl-benzothiazoline-sulfonic acid
AGID	Agar gel immunodifusion
BDSL	Biological Diagnostic Supplies Ltd.
bp	Base pair
CDC	Center for Diseases Control and Prevention
CFT	Complement fixation test
DVTD	Department of Veterinary Tropical Diseases
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization
HI	Haemaglutination inhibition
I-ELISA	Indirect enzyme-linked immunosorbent assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
OD	Optical density
OIE	Office International des Epizooties – World organisation for animal health
PBS	Phosphate buffered saline
PBS+	Phosphate basic solution of Dulbecco
PCR	Polymerase chain reaction
PP value	Percentage of positive value
RVF	Rift Valley fever
recN I-ELISA	Recombinant- nucleocapsid enzyme-linked immunosorbent assay
SDS	Sodium dodecyl sulfate
SNT	Serum neutralization test
SOP	Standard operation procedure
VN	Virus neutralization
WHO	World Health Organization

SUMMARY

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During March 2008 a suspected outbreak of Rift Valley fever was reported on a farm in the Bela-Bela area, Limpopo Province of South Africa. The affected dairy farm, where no vaccination programme against RVF were practiced, applies an intensive farming system with 300 Holstein Friesland cattle (calves included) as well as 200 Pedi sheep on the farm. Seven calves died on this farm but no apparent clinical disease was reported in cattle as well as in sheep. During the outbreak blood samples from cattle and sheep were taken and the animals were re-sampled 8 weeks later. A set of sera was also collected from cattle on a neighbouring farm. The aim of the study was to determine the extent of the outbreak by evaluating if the virus had also infected other animals on the affected farm as well as on a neighbouring farm.

During the first blood collection 233 samples were taken from cattle and 73 from sheep on the affected farm; 55 blood samples were taken from cattle on a neighbouring farm. A second blood collection was only done on the affected farm and 234 cattle and 85 sheep were bled. All the sera collected were tested by an IgM-capture ELISA and by an

indirect IgG ELISA. Selected IgM positive (n=14), IgG positive (n=23) and samples negative for both IgM and IgG (n=19) were then tested by the serum neutralization test (SNT). Sera from IgM positive (14) and negative (20) animals were also tested by a TaqMan PCR.

Results from the affected farm showed that 7% (16/233) of cattle samples were IgM-positive and 13.7% (32/233) IgG positive at the first collection of samples, and 2% were IgM-positive at the second sample collection. The number of cattle positive for RVF virus-specific IgG antibodies increased by 20.3% when compared to the first bled. Only 1.4% of sheep were both positive for anti-RVF virus IgM and IgG antibodies at the first collection; IgM-positive cases decreased to 1.2%, while IgG-positive cases increased to 2.4% at the second bled. Although no IgM-positive cattle could be found on the neighbouring farm, 5.5% of cattle were IgG-positive.

The SNT confirmed most of the ELISA results. Three samples that tested positive for anti-RVF virus IgM and one anti-RVF virus IgG positive sample using ELISA tested negative using the SNT. Two samples that tested negative for both IgM and IgG antibodies using ELISA, tested low positive (1:10 and 1:20) using the SNT. All samples tested using a TaqMan PCR were negative.

On the affected farm, apart from the seven calves that died, cattle were also infected. There was evidence of virus circulation on the neighbouring farm but the negative PCR results indicate that at the time the animals were sampled they were not viraemic.

How the virus was introduced onto the farm is not clear. The possibility of low level virus circulation in animals and the reactivation of virus from endemic foci by the breeding of vector competent mosquitoes on the low-lying area on the farm in Bela-Bela may have led to ideal circumstances for an outbreak to occur. The fact that mostly cattle seroconverted suggests a higher host preference of the local population of mosquitoes for cattle rather than sheep.

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CHAPTER I
LITERATURE REVIEW

1.1 Introduction

Rift Valley fever (RVF) is a peracute to acute mosquito-borne viral disease, affecting mainly domestic ruminants and is characterized by high rates of abortion and neonatal mortality, necrotic hepatitis and a haemorrhagic diathesis. The disease is more severe in new-born lambs and calves than in adults and sheep is more susceptible than cattle and goats (Coetzer 1977; Gerdes 2004). Rift Valley fever is a zoonotic disease and infections in humans often present as an influenza-like illness; less than one per cent of cases develop complications such as a haemorrhagic state and encephalitis that may proceed to death of the patients (Meegan 1979; Balkhy & Memish 2003). Apart from serious consequences for human and animal health, RVF epidemics also affect the international livestock trade (Clements *et al*. 2007).

The disease is caused by RVF virus (Bishop & Shope, 1979). Based on clinical manifestations, RVF virus is placed in the group of RNA viral haemorrhagic fever (VHF) agents that includes ebolaviruses, marburgviruses, Lassa virus, Crimean-Congo Haemorrhagic fever virus, Yellow fever virus, Dengue virus and hantaviruses (Bouloy 1991; Pépin *et al.* 2010). The virus is transmitted biologically by mosquitoes such as *Aedes* and *Culex* spp. (Davies & Highton 1980), but biting flies such as stomoxids and tabanids, midges, phebotomids and simulids (Hoch *et al.* 1985) feeding on viraemic animals may also transmit the RVF virus mechanically but not biologically (FAO 2003).

In central, eastern and southern Africa RVF outbreaks generally occur when climatic conditions favour the breeding of mosquito vectors (Davies *et al.* 1985b; Sall *et al*. 1998; Andriamandimby *et al.* 2010). Until 1975, large outbreaks, with significant health and socio-economic impact, have historically been restricted to sub-Saharan Africa. In 1977 the virus spread to Egypt in North Africa (Meegan *et al*. 1979) and in 2000 it was reported in livestock and humans in Saudi Arabia and Yemen (Balkhy & Memish 2003; FAO 2003).

A suspected or provisional diagnosis of RVF is usually made on the prevailing epidemiological situation, clinical signs and necropsy findings. The provisional diagnosis must be confirmed by virus isolation (Swanepoel & Coetzer 2004), detection of the antigen or viral nucleic acid in whole blood, serum or organs (Viljoen *et al*. 2005; Pestana *et al.* 2010). In this study, serum samples were collected from cattle and sheep on a farm near Bela-Bela (Limpopo Province) with the aim of determining the extent of the outbreak.

1.2 Aetiological agent

Rift Valley fever virus is classified in the genus *Phlebovirus* of the family *Bunyaviridae* (Bishop & Shope 1979; Bouloy 1991). Viruses in this group contain a negative-sense, single-stranded RNA with a tri-partite segmented genome, namely a large (L), medium (M) and small (S) segment (Bouloy 1991; Levy *et al.* 1994).

The S segment uses an ambisense strategy and codes for two proteins: the viral nucleoprotein N and a nonstructural protein NSs. The NSs is synthesized in viral sense (5' to 3') whereas the N protein in viral antisense (3' to 5') (Gentsch & Bishop 1978; Suzich *et al.* 1990; Vialat *et al.* 2000). The L segment encodes for the RNA dependent RNA polymerase L protein (Bouloy 1991; Sall *et al.* 1999). The M segment codes for a polyprotein that is processed to form two envelope glycoproteins (G1 and G2) which then give rise to generate two additional nonstructural proteins of 78 and 14 kDa (Gentsch & Bishop 1979; Kakach *et al*. 1989; Suzich *et al.* 1990; Sall *et al.* 1999). The genome segments are concealed within a spherical virus particle measuring 90 to 100 nm in diameter that is surrounded by an envelope containing glycoprotein surface projections (Murphy *et al.* 1999).

Rift Valley fever virus is sensitive to acid conditions below pH 6 and is readily inactivated by acetone at -30℃, by a 0.25% solution of 10% commercial formalin at 4℃ for 3 days, methylene blue in the presence of light and by lipid solvents such as ether and sodium deoxycholate (Peters & Meegan 1981; Shimshony & Barzilai 1983; Murphy *et al.* 1999). The virus is preserved by temperatures lower than -60℃ or freeze-drying and it is very stable in serum stored at 4℃ for several months or for three hours at 56℃ (Peters & Meegan 1981). The virus is stable at environmental temperature of 25-30℃ for 80 minutes (Balkhy & Memish 2003).

1.3 Epidemiology

1.3.1 Hosts

Rift Valley fever was first described as a fatal disease of sheep due to the high incidence of abortion in ewes and a high mortality in lambs in the Rift Valley in Kenya (Findlay & Daubney 1931). The disease also occurs in cattle and goats (Findlay 1931) but the disease in these species is usually less severe than in sheep (Gerdes 2004). The mortality rates in new-born lambs can exceed 90%. In new-born calves the mortality rate is about 10-20% (Coetzer 1977; Swanepoel & Coetzer 2004). Although some reports indicate that some African indigenous livestock breeds of sheep, goats and cattle are less susceptible to clinical disease than exotics breeds (FAO 2003, OIE 2009), others are of the opinion that indigenous livestock are just as susceptible as exotic breeds (Swanepoel & Coetzer 2004).

The disease has also been reported in a range of wildlife species such as African buffaloes (*Syncerus caffer*), lesser kudu (*Tragelaphus strepsiceros*), Thomson's gazelle (*Gazella thomsonii*), impala (*Aepyceros melampus*), black rhino (*Diceros bicornis*), waterbuck (*Kobus ellipsiprymnus*), sable (*Martes zibellina*), and springbuck (*Antidorcas marsupialis*) (Swanepoel & Coetzer 2004, Gerdes 2004; Evans *et al.* 2008). No antibodies to RVF virus were detected in lions (*Panthera leo*), zebras (*Equus burchelli*), giraffes (*Giraffa camelopardalis*) and warthogs (*Phacochoerus aethiopicus*), suggesting that these species are not susceptible to RVF virus (Evans *et al.* 2008). There are also reports of the disease in camels (*Camelus dromedarius*) (Meegan 1979; Davies *et al.* 1985a).

Laboratory infections have demonstrated that mice, certain small rodents (Findlay 1931), monkeys (*Macacus rhesus*) and cats are susceptible to infection with RVF virus (Findlay & Daubney 1931). Rabbits, guinea-pigs, mongooses and birds are however not susceptible (Findlay & Daubney 1931; Findlay 1931; Henning 1952). Rift Valley fever is also an important zoonotic disease (Findlay 1931; Alexander 1951; Laughlin *et al.* 1979; Paweska *et al.* 2010).

1.3.2 Transmission and maintenance of RVF virus

Two cycles (endemic and epidemic) of RVF transmission have been described (Labeaud *et al.* 2007; Evans *et al*. 2008). The role of mosquitoes as biological vectors in the transmission and maintenance of RVF virus has been outlined by several studies (Davies & Highton 1980; Gad *et al.* 1987). According to Bird *et al*. (2009), RVF virus has been isolated from more than 30 species of mosquitoes belonging to 6 genera, such as *Mansonia, Eretmapodites* (Gerdes 2004), *Culex*, *Aedes*, *Anopheles* (Davies & Highton 1980; Gad *et al.* 1987) and *Coquillettidia.* During the endemic cycle the virus is maintained dormant in mosquito eggs after transovarial transmission (Bird *et al*. 2009). The virus survives for long periods in mosquito eggs in dried mud. After sustained rainfall, the infected eggs hatch and give rise to infected mosquitoes (Linthicum *et al.* 1988; Murphy *et al.* 1999). A number of studies attempting to understand the role of hosts, other than mosquitoes, in the maintenance of the virus during inter-epidemic periods, have been published. Serological surveys in sheep, goats, cattle and wildlife in southern Africa (Swanepoel & Coetzer 2004) and eastern Africa (Rostal *et al*. 2010) suggested that virus activity occurs during inter-epidemic periods.

Epidemics occur during sustained increased rainfall resulting in an increase of the population of competent mosquitoes and consequently increased virus transmission to susceptible vertebrate hosts (Davies *et al.* 1985b; Acha & Szyfres 1989). After infection, sheep and cattle develop a high viraemia of about one week's duration and act as virus amplifiers. Mosquitoes become infected when feeding on viraemic animals and can then act as vectors when feeding on non-infected animals (Jupp *et al.* 1984). On the other hand, some infected mosquitoes maintain the virus during inter-epidemic periods by transovarial transmission (Linthicum *et al*. 1988).

Rift Valley fever is a zoonotic disease (Findlay & Daubney, 1931; Andriamandimby *et al.* 2010) and humans acquire infection through exposure to aerosols generated by blood and tissues when opening a carcass of an infected animal (Anyangu *et al*. 2010) such as slaughtering and skinning of animals, and handling of aborted foetuses (Henning 1952; Labeaud *et al*. 2008; Anyangu *et al.* 2010). These are particularly important during large outbreaks in which many animals are infected; their blood and organs, including the tissues of aborted foetuses, contain high concentrations of the virus (Findlay & Daubney 1931; Gonzalez-Scarano *et al*. 1991; Geering & Davies 2002; Labeaud *et al*. 2008). Viraemic animals shed the virus in milk but pasteurization

inactivates the virus (Acha & Szyfres 1989), however consumption of raw milk also has been considered a source of infection (Balkhy & Memish 2003).

Rift Valley fever cases in humans are always linked to a disease outbreak in livestock (Alexander 1951; Paweska *et al*. 2010). Surveys carried out in the human population during the RVF outbreaks in Kenya in 1997-98 (Woods *et al*. 2002) and in Madagascar in 2008 (Andriamandimby *et al.* 2010) reported evidence of human infection, but in both studies retrospective investigations showed that they had been exposed to animals.

The possibility of RVF transmission to humans by the bite of a mosquito has been reported (Gonzalez-Scarano *et al.* 1991; Gerdes 2004; Andriamandimby *et al.* 2010). There is, however, no report of human to human transmission of RVF (WHO 2008). Evidence of inter-epidemic human transmission of RVF virus in Kenya was reported, although the mechanism by which humans became infected during this period is unknown (Labeaud *et al*. 2007; 2008).

The spread of RVF virus into non-endemic areas can therefore be via viraemic animals and mosquito vectors from an infected area (Balkhy & Memish 2003). Most of the outbreaks that occurred in RVF virus-free regions of South Africa as well as Zimbabwe, Sudan, Zambia and Egypt, suggested that some species of mosquitoes from an endemic area introduced the virus. The outbreak in Egypt in 1979 suggested that the virus was introduced by means of viraemic camels, and infected mosquitoes carried from the neighbouring affected Sudan on winds from the intertropical convergence zone (Laughlin *et al.* 1979; Acha & Szyfres 1989).

According to Gonzalez-Scarano *et al.* (1991) the role of the wildlife-mosquito cycles in virus maintenance during the inter-epidemic periods should be considered. The RVF wildlife-mosquito cycle suggests that there is virus transmission between mosquitoes and susceptible wildlife animals in which the latter undergo mild or asymptomatic infections. When conditions (floods) favouring the proliferation of mosquito populations occur, virus amplification takes place with further transmission of virus by competent mosquitoes to livestock (Bird *at al.* 2009).

1.3.3 Occurrence

In South Africa the first outbreak of RVF was recorded and confirmed in 1950, when heavy mortality in sheep and cattle were reported in the western and other areas of the Orange Free State (now Free State Province). The first outbreak of RVF in humans in South Africa was confirmed when the RVF virus was also isolated from blood collected from professionals who performed a necropsy on a bull that died near Johannesburg (Alexander 1951; Libel & Slander 1952). Two hypotheses were considered regarding the introduction of the virus into South Africa: that the virus was introduced by infected mosquitoes brought by air or by infected humans (Alexander 1951).

During 1974-75 South Africa experienced the second big epidemic of RVF in sheep and cattle which was preceded by wet climatic conditions that favour the breeding and subsequent increase in the insect vector population (Coetzer 1977; Coetzer & Ishak 1982). The reports started in March 1974 in the Bultfontein area in the Free State Province and spread to other regions including the North-west of the Cape Province (now Northern Cape Province), South West Africa (now Namibia), Northern Transvaal (now Gauteng Province), Northern Zululand (now KwaZulu-Natal Province and Swellendam district of the Cape Province (now Western Cape Province) (Coetzer 1977). The mortality rates in lambs (90-95%), in calves (10-15%) and sheep (15-20%) were high. A high percentage of the pregnant sheep and cattle aborted during the outbreak (Coetzer 1977).

In 2008, another epidemic of RVF (Figure 1) occurred in livestock (sheep, cattle and goats) and wildlife in South Africa affecting four neighbouring provinces (Mpumalanga, Limpopo, Gauteng and North-West Province). In 2009 outbreaks of RVF were reported in KwaZulu-Natal Province (Figure 2). In contrast to the widespread epidemics of 1950-51 and 1974-75, the 2008 and 2009 outbreaks were limited to certain parts of South Africa (OIE 2010).

A third large epidemic occurred in 2010 in South Africa (OIE 2010) affecting the eastern Free State Province, areas of Northern Cape, Eastern Cape, Gauteng, and Mpumalanga Provinces and the Western Cape Province (Figure 3). Domestic livestock (sheep, cattle and goats) as well as different wildlife species were affected (OIE 2010). In humans 1588 were suspected cases of which 237 were laboratory confirmed and 26 cases were fatal (Paweska *et al*. 2010).

The outbreak in Egypt during 1977-1978 (Laughlin *et al* 1979) called international attention and concern that the virus might spread to the RVF-free regions outside Africa (Meegan *et al.* 1979). Epidemics of RVF were subsequently reported in many other African countries including western African countries such as Gambia, Guinea, Mauritania, Burkina Faso and Niger (Saluzzo *et al.* 1987). Madagascar (Morvan *et al*. 1991; Morvan *et al.* 1992a) recorded RVF outbreaks for the first time in 1991 and large outbreaks in 2008 and 2009 (Andriamandimby *et al*. 2010).

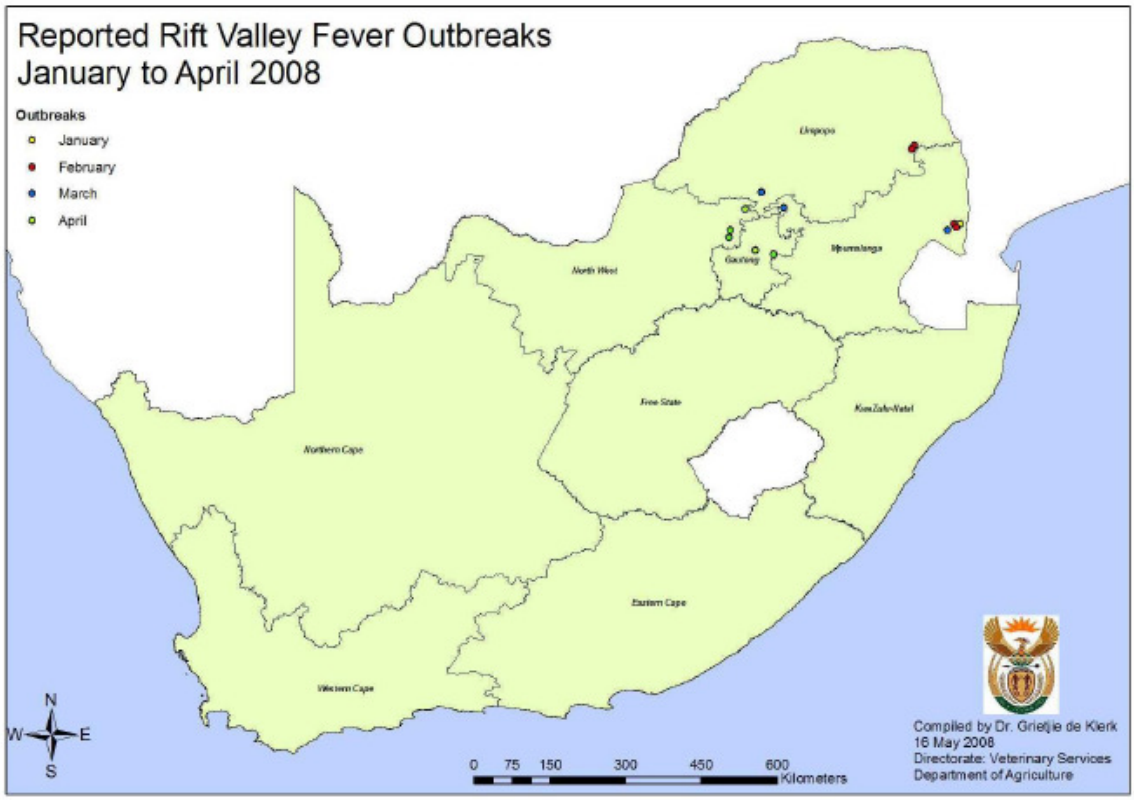

Figure 1 Rift Valley fever outbreaks in animals in South Africa in 2008.

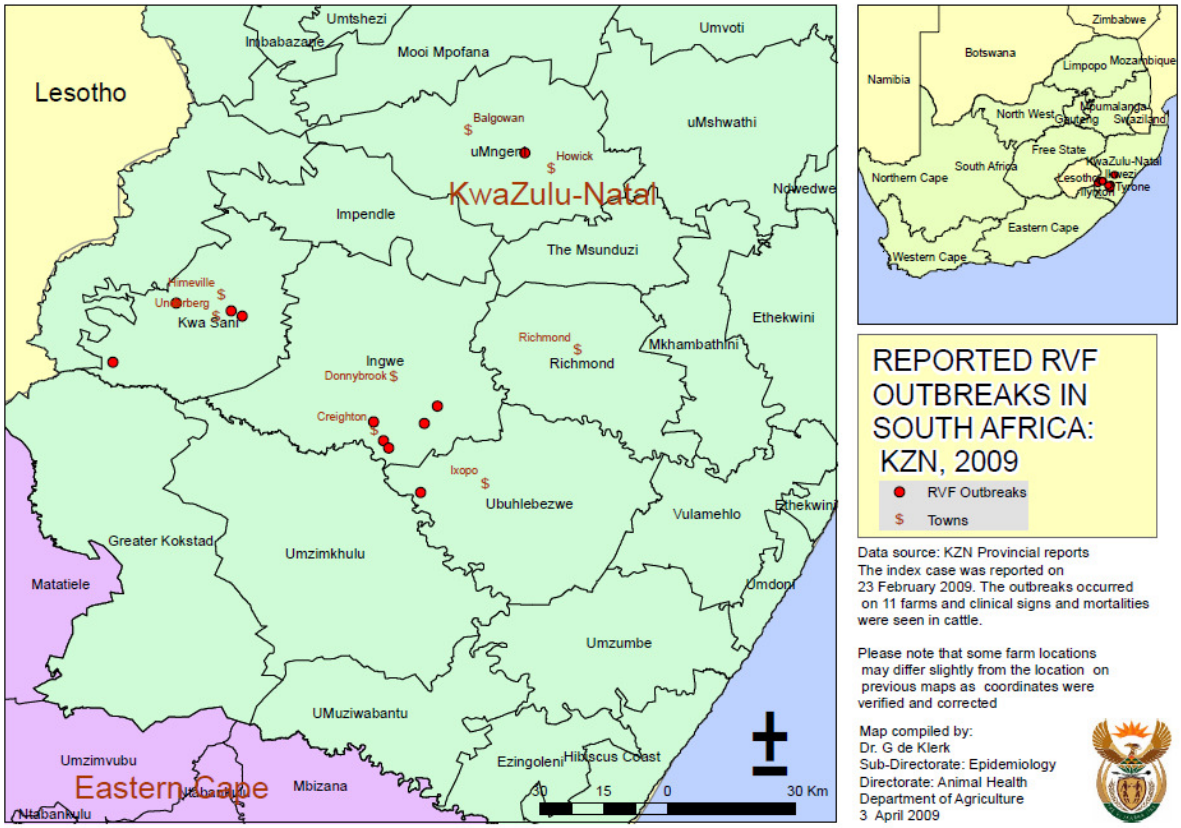

Figure 2 Rift Valley fever outbreaks in animals in KwaZulu-Natal, South Africa in 2009.

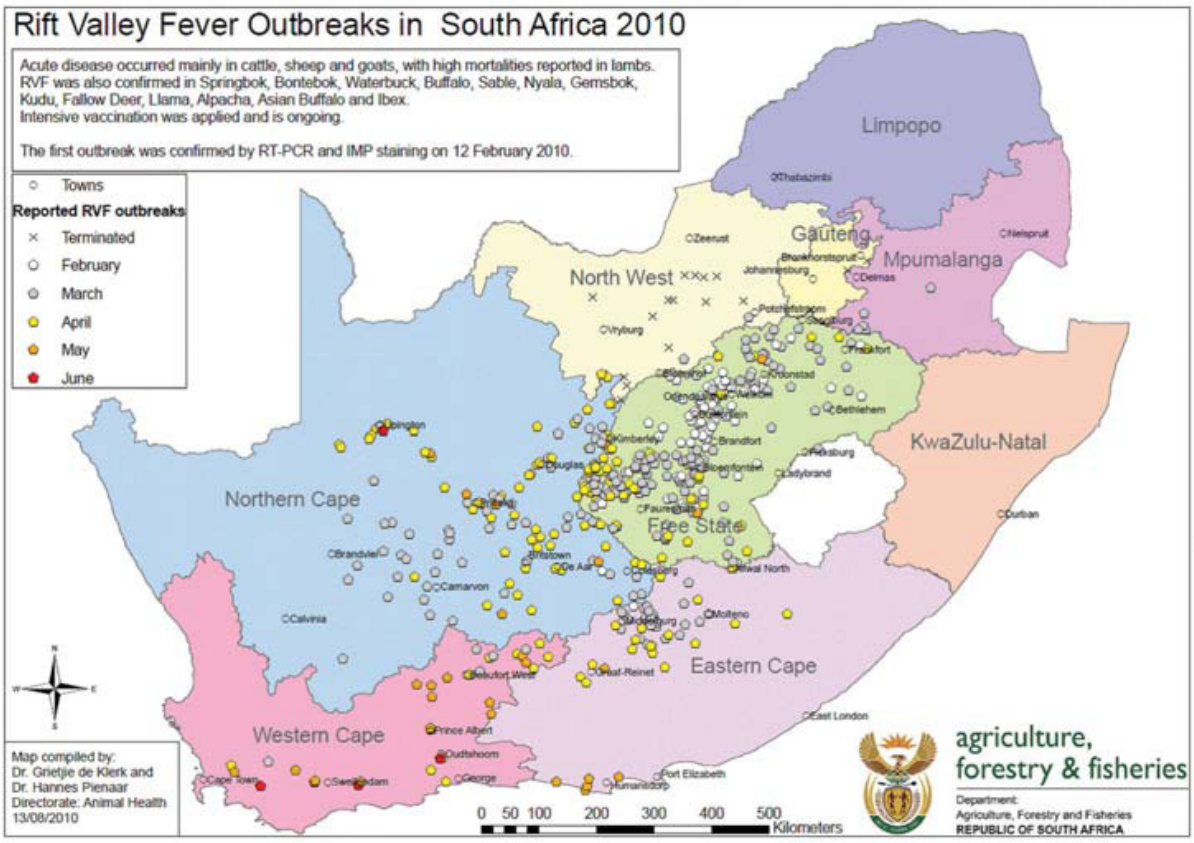

Figure 3 Rift Valley fever outbreaks in animals in South Africa in 2010.

1.4 Diagnosis

A provisional diagnosis of RVF is made when abortion in sheep, cattle and goats associated with deaths in young animals occurs after exceptional heavy rain. Confirmatory diagnosis of RVF is based on histopathology, virus isolation, serology and/or detection of viral nucleic acid (OIE 2009).

The diagnosis of the RVF can be confirmed by demonstrating a rise in anti-RVF virus IgG antibody titres in paired serum samples. The first serum sample should be taken during the acute stage and the second during the convalescent stage (2-3 weeks later). An increase of at least fourfold in antibody titre is indicative of infection (Topley & Wilson 1990). However with the IgM capture ELISA, a single serum sample taken during the acute stage is enough for the diagnosis of acute infections (Paweska *et al*. 2003).

Several serological tests have been used for the detection of anti-RVF virus antibodies, including the virus-neutralization (VN) test, haemaglutination inhibition (HI) test, complement fixation (CF) test, immunodifusion (ID) test, plaque reduction neutralization (PRNT) test, enzyme- linked immunosorbent assay (ELISA), radioimmunoassays (RIA) test and indirect immunofluorescence (IF) (Swanepoel *et al.* 1986). From the above mentioned tests the VN, ELISA, HI, and ID are most commonly used.

Virus isolation is the conclusive method for confirmation of RVF. Many different cell types have been described for the isolation of RVF virus (WHO 1982) and the recommend isolation procedures are described in the OIE Laboratory Manual (2009a). As discussed later, molecular techniques are often used these days to confirm a diagnosis of RVF.

1.4.1 Virus neutralization test

The VN test has been used to detect antibodies against RVF virus in serum of a variety of species, and the OIE considers this test as the gold standard to test for anti-RVF virus antibodies (OIE 2009). The test is highly specific, but it can only be performed with live virus and therefore is not recommended for use outside endemic areas or in laboratories without appropriate biocomtainment facilities and vaccinated personnel (OIE 2009). Most types of cell cultures including African green monkey kidney cells

(Vero), baby hamster kidney (BHK-21) and rhesus monkey kidney epithelial cells (LLC-MK2) can be used in the VN test for RVF (WHO 1982).

1.4.2 Enzyme-linked Immunosorbent assay

ELISA-based assays are very useful diagnostic techniques that can be used to detect either antibody or antigen (Dinter 1989). Antibody ELISA's are widely used due to their high sensitivity, specificity, low costs, safety and large numbers of animals can be tested in short period of time (Paweska *et al*. 2003; OIE 2009). A number of RVF ELISA's have been developed and validated to detect antibodies in cattle, sheep, goats, African buffalo and humans (Paweska *et al*. 2003; Paweska *et al*. 2005; Jansen van Vuren *et al.* 2007; Fafetine *et al.* 2007).

An indirect ELISA (I-ELISA) using an inactivated, cell culture-produced antigen and Protein G-peroxidase conjugate has been developed and extensively tested (Paweska *et al.* 1995). An I-ELISA based on a recombinant- nucleocapsid (recN I-ELISA) antigen for the detection of anti-RVF virus antibodies in domestic ruminants was also developed by Fafetine *et al.* (2007).

The IgG-sandwich and IgM-capture ELISA (both based on inactivated antigen) for the detection of anti-RVF virus IgG and IgM antibodies in cattle, goats and sheep sera were developed and validated (Paweska *et al*. 2003). Compared to the VN and HI tests, the IgG-sandwich ELISA was more sensitive for the detection of early immunological responses to infection of RVF virus (Paweska *et al*. 2003). The RVF IgM-capture ELISA is used in order to detect recent infection/acute stage of an infection or shortly thereafter (OIE 2009). The ELISA's, due to their high sensitivity and specificity and safety, have the potential to replace traditional methods of RVF diagnosis (Paweska *et al*. 2003).

1.4.3 Molecular-based assays

The polymerase chain reaction (PCR) detects viral nucleic acid of RVF virus in blood, serum and tissues (Garcia *et al*. 2001). Several PCR assays for the diagnosis of RVF in humans and animals have been developed (Drosten *et al.* 2003). The real-time reverse transcriptase PCR measure the amount of PCR product when the reaction is still in the exponential phase (Garcia *et al*., 2001; Espach *et al.* 2002; Drosten *et al*. 2002; Sall *et*

al. 2002; Viljoen *et al*. 2005) The real-time reverse transcriptase loop-mediated isotermal amplification in which a target sequence is amplified using two or three sets of primers. The amount of DNA product obtained is high and the costs are low (Peyrefitte *et al*. 2008; Le Roux *et al*. 2009). In the nested reverse transcriptase PCR the template of the first amplification undergo a further amplification and thus allow obtaining more copies of DNA and also increases the specificity as well as the sensitivity of amplification (Sall *et al.* 2001). Molecular tools have also been developed to detect RVF virus in vectors (Ibrahim *et al.* 1997). Drosten and collaborators (2003) summarized the PCR that are available for the diagnosis of RVF virus (Table 1).

TaqMan Reverse Transcriptase Detection PCR (RTD-PCR)

While the traditional PCR is time consuming due to the different steps involved, the real-time PCR, aside from minimising the risk of contamination, is quick as it measures the amount of PCR product while it is amplified, and post PCR analysis on agarose gel is not necessary (Pestana *et al.* 2010). One of the technologies used for real-time detection of RVF virus is Taqman probes (Drosten *et al.* 2002; Bird *et al*. 2007). Taqman probes have high specificity, high signal to noise ratio, and detect multiple sequences in one reaction which is an advantage when the amount of DNA is very low (Pestana *et al.* 2010).

Table 1 Published PCR assays for detection of RVF virus (Adapted from Drosten *et al*. 2003)

PCR	Virus and target region	Method	Primer	Sequence	Sensitivity	Reference
1	RVF G2	Two-steps nested	RVF1	gactaccagtcagctcattacc (ag)	0.5 pfu/PCR	Ibrahim *et al.* (1997)
			RVF2	tgtgaacaataggcattgg (g)		
			RVF3	cagatgacaggtgctagc (nested ag)		
			RVF4	ctaccatgtcctccaatcttgg (nested g)		
2	RVF NSs	Two-steps nested	NSca	ccttaacctctaatcaac (g)	Clinical 70% GS: virus isolation; 0.5pfu/PCR	Sall *et al.* (2001, 2002)
			NSng	tatcatggattactttcc (ag)		
			NS3a	atgctgggaagtgatgagcg (nested g)		
			NS2g	gatttgcagagtggtcgtc (nested ag)		
3	RVF NSs	Two-steps Real-time	S432	atgatgacattagaaggga (ag)	100 copies/PCR	Garcia *et al.* (2001)
			NS3m	atgctgggaagtgatgag (g)		
			CRSS	FAM-attgacctgtgcctgttgcca-TX		
4	RVF G2	One-step Real-time	RVS	aaaggaacaatggactctggtca (ag	95% detection limit: 2835 copies/ml 16 copies/PCR	Drosten *et al.* (2002)
			RVAs	cacttcttactaccatgtcctccat (g)		
			RVP	FAM-attgacctgtgcctgttgcca-TX		

Abbreviations: g: genomic primer, **ag**: antigenomic primer; **GS**: gold standard for determining clinical sensitivity, **PFU**:plaque forming units, **FAM**: 6-carboxyfluorescein, **TX**: 6-carboxytetramethylrodamine, G2: glycoprotein 2.

1.5 Problem

In March 2008 an outbreak of RVF was reported in Bela-Bela, Limpopo Province of South Africa. Seven dead calves were reported on one farm. Notwithstanding the fact that the animals on the affected farm and a neighbouring farm were not vaccinated against RVF, no clinical disease was reported in any of the other animals on the affected and neighbouring farms. The absence of clinical disease in the other animals (cattle and sheep) on the affected farm and in cattle on the neighbouring farm, called attention to the need for exploring this rather unusual pattern of the disease.

1.6 Objectives

The aim of this study was to determine the extent of the outbreak by:

- Determine the level of anti-RVF virus antibodies in cattle and sheep on the affected farm as well as in cattle from a neighbouring farm using an I-ELISA and SNT, and to
- Determine virus circulation on the two farms by using a real-time RT-PCR for detection of RVF virus-specific nucleic acid in serum samples.

CHAPTER II
MATERIALS AND METHODS

2.1 Background and description of outbreak

On 10 March 2008 the Section of Pathology, Faculty of Veterinary Science, University of Pretoria received 7 dead calves (one week to two-months-old) from a farmer in the Bela-Bela district, Limpopo Province, South Africa. Post-mortems were done and Rift Valley fever expected. Serum samples were then collected on the affected and neighbouring farms by the State Veterinarian of the region as well as veterinary students of the Faculty of Veterinary Science, University of Pretoria. Samples were collected from 77.7% (233/300) of cattle and 36.5% (73/200) of sheep on the affected farm as well as from cattle (n=55) on the neighbouring farm. To evaluate sero-conversion as well as active infections, 78.0% (234/300) of cattle and 42.5% (85/200) of sheep from the affected farm, were bled again eight weeks after the first collection.

The affected dairy farm applies an intensive farming system with 300 Holstein Friesland cattle (calves included) as well as 200 Pedi sheep on the farm. Animals were kept in different camps. The total number of calves was 40 and they were kept in cages inside a building. The cages were lifted from the ground and were positioned in such a way that the afternoon sun was shining onto the cages through the only opening of the building. The lights in the building were kept on during the night. The sheep were kept in a camp about 200 m away from the calves and the adult cattle about 100 m away, forming a triangle. All the calves that died were born on the affected farm and there was no history of newly introduced animals. The farmer did not practice any vaccination programme.

Seven of the calves were found dead one morning (10 March), after they have not shown any signs of illness having drunk their milk the previous evening. Subsequent to these deaths none of the other animals showed clinical disease, no visible change in milk production and no abortions or further mortalities were reported.

The vegetation of Bela-Bela, consists of grasslands and trees which is generally referred to as bushveld. Rainfall data in the area indicated that rainfall for January 2008 was more than 100 mm above the average monthly rainfall for that month. Rainfall in February 2008 was below the average monthly rainfall and rainfall in March was normal for that time of the year (South African Weather Services).

The geographical coordinates of the farm in the Bela-Bela district where the outbreak occurred are: E 28º 19` 28" and N 24º 51`10". Approximately one kilometer from the area where the cattle and sheep are housed is a low-lying area with a water stream which flow from Klein Kariba, a small holiday resort close to Bela-Bela. This swampy area has a dense plant growth with a lot of cane. The neighbouring farm, whose cattle were bled for this study, is in this same direction and a tar road is separating it from the affected farm. There are no other farms and no other cattle in close proximity to the affected farm. None of the farm workers, who assisted in the transport of the dead animals showed any clinical signs of RVF. The farmer however became very sick showing flu-like symptoms.

2.2 Sample collection

Blood samples collected from the two farms in the Bela-Bela district, were from animals of both sexes and different ages. During the first collection which took place on 19 and 20 March 2008, cattle and sheep from the originally affected farm and cattle from a neighbouring farm were bled. The second collection took place from cattle and sheep only on the affected farm between 13 and 16 May 2008, when 234 cattle and 85 sheep were sampled. The serum of each sample was separated after the blood had clotted, and serum samples were labelled / identified according to the animal number, animal species and the farm on which they were collected.

2.3 Laboratory tests

All the samples collected were tested using an IgM-capture ELISA and an indirect IgG ELISA supplied by the Special Pathogen Unit of the National Institute for Communicable Diseases (NICD) of the National Health Laboratory Services (NHLS). Based on ELISA results, selected IgM positive (n=14), IgG positive (n=23) and negative for both IgM and IgG (n=19) samples were then tested with the SNT. Selected ELISA IgM-positive sera

(n=14) and negative sera for both by IgM and IgG ELISA (n=20) were tested by a TaqMan PCR assay.

2.3.1 IgM-capture ELISA

The sera were first inactivated at 56℃ for 30 minutes in a water bath before performing the ELISA. The IgM-capture ELISA was conducted at the SPU of the NICD-NHLS, following the procedures described by Paweska *et al*. (2003), as follow:

The ELISA plate (MaxiSorb plates from Nunc, Denmark) was coated with 100 µl of capture antibody (rabbit anti-sheep IgM) diluted 1:500 in phosphate buffered saline (PBS) and incubated overnight at + 4℃. After washing with wash buffer (0.1% of Tween 20 in PBS), the plate was blocked by adding 200 µl of blocking buffer (10% of skimmed milk in PBS) and incubated for 1 h in a moist chamber at 37 ℃. The plate was washed followed by the addition of 100 µl in duplicate of test and control sera diluted 1:400 in diluent buffer (2% skimmed milk in PBS) and incubated in a moist chamber at 37℃ for 1 h. After washing, 100 µl of RVF virus antigen diluted 1:400 were added to the wells in rows A-D: 1-12 (the top half of the plate) while 100 µl of the control antigen diluted 1:400 were added to those in rows E-H: 1-12 (the bottom half of the plate) and the plate was incubated for 1 h in a moist chamber at 37℃. After the washing step, 100 µl of the secondary antibody (mouse anti-RVF virus serum) diluted 1:2000 were added followed the incubation. The plate was washed and 100 µl of anti-mouse IgG HRPO conjugate at a dilution of 1:6000 were added. The plates were incubated for 1 h in a moist chamber at 37℃. After washing the plates 6 times, 100 µl of 2,2` -azino di-ethyl-benzothiazoline-sulfonic acid (ABTS) were added and the plates left at room temperature in the dark for 30 minutes. The stop solution (1% sodium dodecyl sulfate – SDS) was added and the optical densities (OD) were measured at 405 nm using a Elx 800 Universal Microplate Reader (Bio-TeK Instrument, INC).

In each case the washing of plates was performed 3 times (except the last wash) with 300 µl of wash buffer for 15 seconds, using ELx 405 Auto Plate Wash (Bio-TeK Instrument, INC). The raw ODs were converted to PP values using the following formula:

$$PP = \frac{\text{Net OD serum (C+,or C-, or test serum)}}{\text{Net mean OD C++}} \times 100$$

PP = percentage of positive; OD = optical density; C+ = weak positive control serum; C- = Negative control serum; C++ = higher positive control serum.

Sheep sera which produced PP values ≥ 8 PP and cattle sera which produced PP values ≥ 14.3 were considered to be positive; PP values below bellow these values were considered to be negative (Appendix 4) (Paweska *et al*. 2003).

2.3.2 recN IgG indirect ELISA

The recN IgG indirect ELISA was conducted at the SPU of the NICD-NHLS, following the procedures described by Jansen van Vuren *et al*. (2007). The ELISA plates (MaxiSorb plates from Nunc, Denmark) were passively coated with 100 µl of RVF virus recN antigen diluted 1:2000 in coating buffer (Carbonate-Bicarbonate) and incubated at 4℃ overnight. The plates were washed with PBS and 200 µl of blocking buffer were added. After incubation for 1 h in a moist chamber at 37℃, the plates were washed with PBS and 100 µl of test or control sera at a dilution of 1:400 were added in duplicate and incubated again for 1 h in a moist chamber at 37℃. After washing the plates with PBS, 100 µl of protein G HRPO conjugate at a dilution of 1:10000 were added per well. Plates were incubated at 37℃ for 1 h and washed with PBS. A volume of 100 µl of ABTS substrate was then added to each well. The plates were incubated in the dark at room temperature for 30 minutes and 100 µl of SDS were added to stop the reactions. The OD was determined using a 405 nm filter. The raw ODs were converted to PP values using the formula:

$$PP = \frac{\text{Mean OD serum (C+, or C-, or Test serum)}}{\text{Mean OD C++}} \times 100$$

The sera which produced PP values ≥ 30 PP were considered to be positive; values lower than those were considered to be negative (Appendix 3). After each step plates were washed 3 times using ELx 405 Auto Plate Wash (Bio-TeK Instrument, INC an ELISA plate washer) (Jansen van Vuren *et al*. 2007).

2.3.3 Serum-neutralization test

The serum-neutralization test was done at the Virology Section of the DVTD according to the Standard Operation Procedures (SOP) in use in this section. The test was performed in 96-well microtitre plates and a vaccine strain of RVF virus (obtained from Onderstepoort Biological Products) was used as antigen.

The sera tested were diluted 1: 5 in phosphate buffered saline of Dulbecco (PBS+) and inactivated for 30 min in a water bath at 56°C. Two-fold dilutions of the serum were prepared in duplicate on the microtitre plate (1-6 wells) and 100 µl of each dilution were used. The RVF stock virus with a titre of $10^{4.6}$ $TCID_{50}$/100 µl was diluted in MEM containing 5% fetal calf serum to provide $100TCID_{50}$. A volume of 100 µl was added to each well that contained a diluted test serum. To determine if the correct concentration of the virus was used, a series of ten-fold dilutions of the virus (10^{-1} – 10^{-4}) were prepared. This virus control (back titration) was used in 3 wells. The plates were incubated at 37°C for 1 h in an incubator containing 5% CO_2. A volume of 80 µl of Vero cells at a concentration of 480 000 cells/ml was added to each well of the microplate including the virus controls, followed by incubation at 37°C in an atmosphere containing 5% CO_2. The plates were read once the virus controls showed 100% cytopathic effect (CPE), 10^{-1} shows 75% CPE, 10^{-2} shows 50% CPE and 10^{-3} show 25% CPE. It is important that 10^{-2} shows 50% CPE because this is the same as the $TCID_{50}$, using 6 wells as follows: 100 µl of virus dilution as used in the test ($100TCID_{50}$) were placed into wells one and two, while 100 µl of each of the ten-fold dilutions were placed in wells three to six.

The monolayers were examined daily from day three until the dilution of 10^{-2} showed 50% CPE. Evidence of CPE was determined by using an inverted microscope and the results were recorded as the last dilution where no inhibition of CPE of the virus could be seen (Appendix 5).

2.3.4 TaqMan Polymerase chain reaction

RNA extraction

The PCR was carried out at the SPU of the NICD-NHLS. Viral RNA was extracted from 140 µl IgM-positve sera as well as from sera negative for anti-RVF virus-specific IgM and IgG antibodies. The QIAamp® Viral RNA Mini kit (QIAGEN, Germany) was used

according to the manufacture`s protocol. The extracted RNA was eluted in 60 µl of buffer AVE, obtained from the kit, and stored at -20℃ until use. One RVF positive sheep serum from a previous study was included in the extractions. This serum had been inactivated and stored at +4℃ for more than one year.

TaqMan reverse transcriptase detection PCR

The Taqman probes consist of a single stranded oligonucleotide which is complementary to a sequence within the target template and their mode of action is explained by Pestana *et al.* (2010) as follows: The probe has a fluorescent dye at its 5' end whose signal is silenced by a quencher dye molecule, the Black Hole Quencher, at the 3' end. Taqman probes use a fluorescence resonance energy transfer as a quenching device so as long as the quencher and the fluorescence dye are in close proximity, quenching will occur. Soon after the Taqman probe hybridizes to one of the strands on the template it is digested by the 5' exonuclease activity of the Taqman DNA polymerase as it extends the amplification primers. Cleaving of the probe releases the fluorescence dye from the quencher resulting in an irreversible increase in the fluorescence signal. Fluorescence will increase as the PCR cycles progress, proportional to the rate of probe cleavage and ideally proportional to the rate of amplified DNA.

The TaqMan reverse transcriptase detection PCR assay was carried out as described by Drosten *et al*. (2002), using the LightCycler RNA amplification kit HybProbe (Roche Diagnostics, Germany) and the LightCycler instrument (Roche). Amplification was carried out in a total volume of 20 µl reaction mixture containing 5 µl of the target virus RNA or the *in vitro* transcribed RNA standards, 1 µM each of the sense (RVS) and antisense (RVAs) primers, 1 µM of probe (RVP) and 5 mM $MgCl_2$.

The primers used target a 94 base pair region of the G2 gene of the M segment of the RVF virus. The sequence of the sense primer (RVS) is 5' AAAGGAACAATGGACTCTGGTCA 3' base pair [349-371]) and that of the antisense primer (RVAs) is 5' CACTTCTTACTACCATGTCCTCCAAT 3' [443-417]). The sequence of the Taqman probe (RVP) 5'- nuclease probe labelled with 6-carboxyfluorescein at the 5' end and with 6-carboxy-N,N,N',N'-tetrame- thylrhodamine at the 3' end, is 5' AAAGCTTTGATATCTCTCAGTGC CCCAA [388-416].

The cycling profile was: Reverse transcription at 45°C for 30 minutes, initial denaturation at 95°C for 5 minutes, and 45 cycles with 95°C for 5 seconds and 57°C for 35 seconds (Drosten *et al.* 2002). Fluorescence was read at the combined annealing-extension step at 57°C.

The difference of proportions of animals positive for anti-RVF virus IgM or IgG antibodies between the two bleeds was compared using the Chi-square test.

CHAPTER III
RESULTS

3.1 Case study findings

On 10 March 2008, seven dairy calves (one week to two-months-old) died on a farm in the Bela-Bela district. A grayish diarrhoea was observed in some of the calves prior to death. No other deaths or clinical disease was reported in adult cattle (n=300) as well as in sheep of the same farm. Five of the carcasses were submitted to the Pathology Section at the Faculty of Veterinary Science, University of Pretoria for post-mortem examination and the findings included multifocal, miliary whitish-grey foci of necrosis with or without haemorrhage scattered throughout a swollen, congested, friable liver. The lungs were diffusely congested and slightly consolidated, and were oedematous. Other lesions included hydrothorax, ascites, hydropericardium, peripheral and visceral lymph nodes enlargement and haemorrhage.

An immunoperoxidase assay revealed specific and strongly positive staining in necrotic and apoptotic hepatocytes, in cells in the alveolar walls in some sections of the lungs and in cells in the red pulp of the spleen.

3.2 ELISA

Of the 233 cattle sera collected during the first collection on the affected farm, 7% (16/233) were IgM-positive and 13.7% (32/233) IgG-positive. Of the cattle that were IgM-positive on the first collection, 56.3% (9/16) were also IgG-positive. During the second collection on this farm IgM antibodies could be found in 2.1% (5/234) of cattle sera. It was also found that 80% (4/5) of cattle IgM-positive also tested positive for IgG antibodies. There was an increase of cattle IgG-positive from 13.7% on first collection to 35% (82/234) on the second collection (Figure 4).

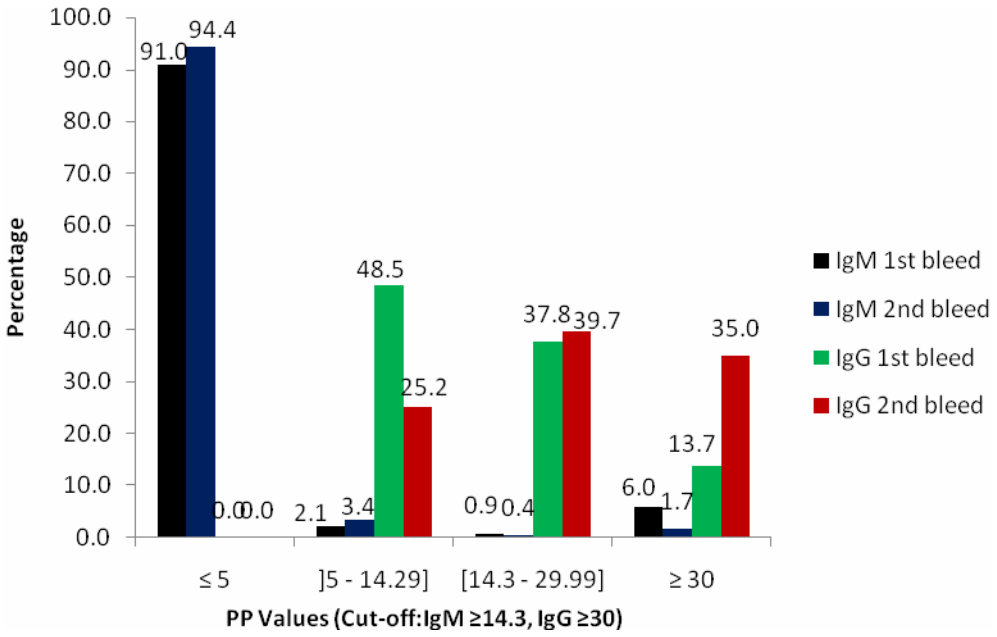

Figure 4 ELISA results obtained from cattle samples after the two collections of blood on the affected farm. For IgM only PP values equal or more than 14.3 were considered positive while for IgG samples that produced PP values equal or higher than 30.0 were considered positive.

Only 1 (1/73) sheep was positive for IgM antibody and another sheep (1/73) IgG antibody during the first bleed. On the second bleed, again only 1 (1/85) sheep (not the same animal as in the 1st bleed) was positive for IgM antibody while IgG antibody was found in two other sheep (2/85) (Figure 5).

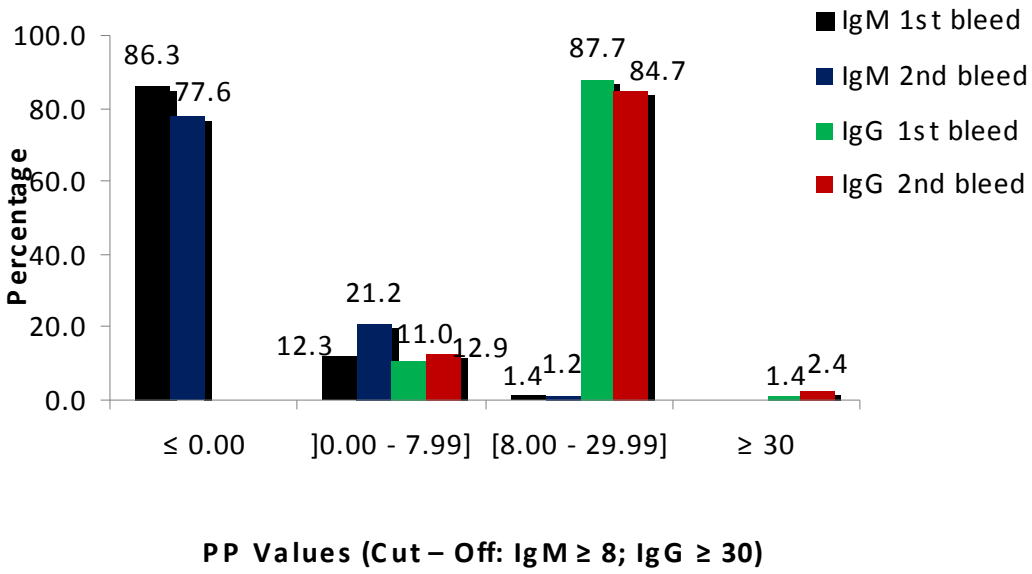

Figure 5 ELISA results obtained from sheep samples after the two collections of blood on the affected farm. For IgM only PP values equal or more than 8.0 were considered positive while for IgG samples that produced PP value equal or higher than 30.0 were considered positive.

Although no IgM-positive results could be found in animals sampled on the neighbouring farm, 5.5% (3/55) of cattle were IgG-positive (Figure 6).

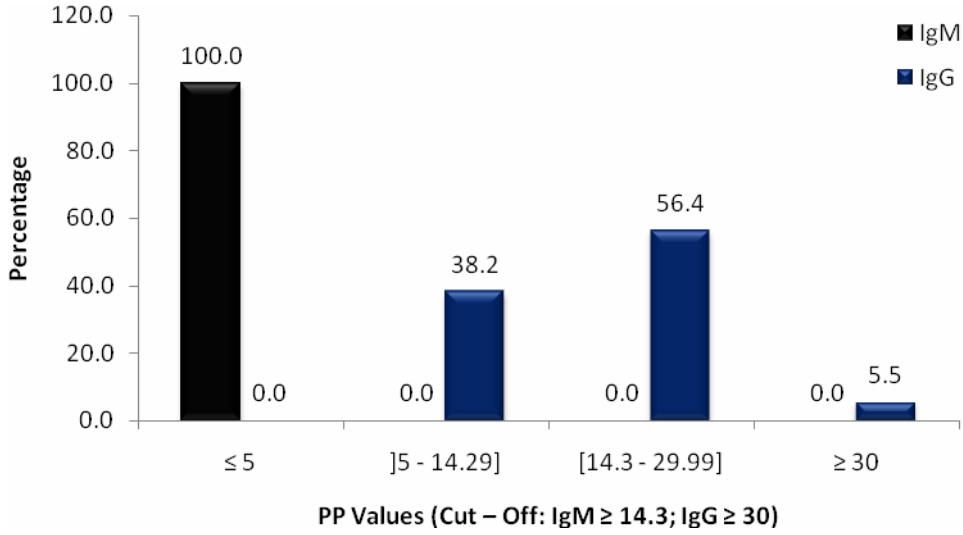

Figure 6 ELISA results obtained from cattle samples after a single collection of blood on the neighbouring farm. For IgM only PP values equal or more than 14.3 were considered positive while for IgG samples were considered positive that produced PP value equal or higher than 30.0.

3.3 Serum-neutralization test

Serum samples that tested positive for either IgM or IgG using the capture and indirect ELISA's were used in the SNT. Three samples that tested positive for IgM and one IgG positive sample using ELISA (all had borderline PP values) tested negative using the SNT. Two samples that tested negative for both IgM and IgG antibodies using ELISA, tested low positive (1:10 and 1:20) using the SNT (Table 2, Appendix 5).

Table 2 Results obtained from selected IgM and IgG positive and negative sera using the serum neutralization test

		Capture ELISA IgM		**Indirect ELISA IgG**	
		Positive (N=14)	Negative (N=19)	Positive (N=23)	Negative (N=19)
SNT	Positive	11	2	22	2
	Negative	3	17	1	17

3.4 Polymerase chain reaction

All sera (n=34) for sheep (n=1) and cattle (n=33) tested by the TaqMan RTD-PCR were negative, however, a PCR positive sheep serum from a previous experiment was included in this study as a positive control during RNA extraction, referred to in Figure 7 as number 25, tested positive (Figure 7). Three standard controls were also included: high positive (Nr. 20), low positive (Nr. 21) and a negative control (Figure 7).

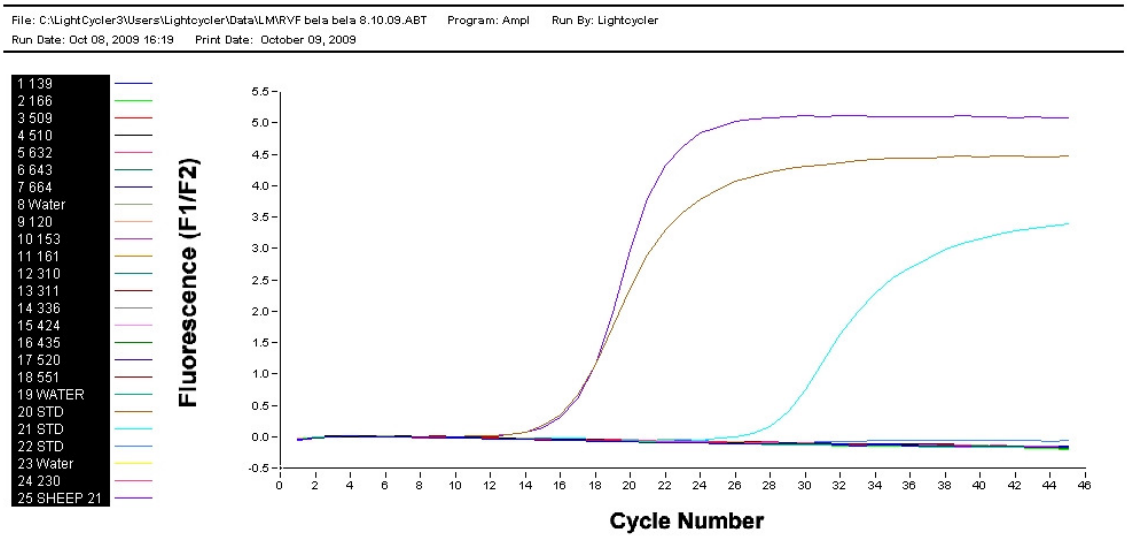

Figure 7 Detection and quantification of RVF virus in suspected sera (sheep and cattle) samples by real-time PCR using Taqman probes. The graphs show the real-time detection of the specific PCR products by fluorescence. Three standard controls were included: high positive (Nr. 20), low positive (Nr. 21) and a negative control. A known positive serum from a previous experiment (Nr. 25) was also included.

CHAPTER IV
DISCUSSION AND CONCLUSIONS

Outbreaks of RVF (n=13) were reported in South Africa in 2008 by the South African Veterinary Services, Department of Agriculture, Forestry and Fishery occurring from January to June which affected cattle, goats, sheep and African buffaloes, in four neighbouring Provinces of South Africa, namely: Mpumalanga (four), Limpopo (three), Gauteng (three) and North-West Province (three) (OIE Ref: 7083, Report Date: 27/05/2008). All these outbreaks were isolated outbreaks of which at least 9 outbreaks including the one at Bela-Bela, described in this study, occurred approximately in a radius of 200 km from each other.

The outbreak in the Bela-Bela district affected one farm. A herd of 300 cattle including 40 calves were present on the farm. Seven of these calves died and macroscopic and histopathological findings obtained from five carcasses were in agreement with findings previously described by Coetzer (1977) as characteristic for RVF virus. Deaths were only reported in calves: 7/40 calves (17.5%) died on 10 March 2008 and no clinical disease, abortions or deaths were reported in adult cattle or sheep also present on the farm. The high mortality rate in calves was also described during the 1976 RVF outbreak in Sudan, where the mortality rate in calves was 40% (Eisa *et al*. 1980) while 10-15% of neonatal mortality was reported during the 1977 RVF outbreak in South Africa (Coetzer 1977).

As the animals were not vaccinated, the presence of IgG is indicative of natural infection. These serological results suggest that the adult cattle from the affected farm also became infected but showed no clinical signs, abortions or deaths were reported in them. These confirm previous reports that most infections in adult cattle with RVF virus are sub-clinical (Findlay 1931; Eisa *et al.* 1980; Davies *et al*. 1992).

It is difficult to speculate when the outbreak first started. The calves died on 10 March and according to time period of viraemia, the OIE (OIE Ref.7083) suggests that the disease started on 1 March 2008. Sera for this study were collected 19 days after the start of the disease (19 March) that gave enough time for sero-conversion to develop. In a study conducted by Morvan *et al.* (1992b), IgM antibodies could still be detected six

months after natural infection, but only 27% (53/195) of these animals were IgM positive by the second month. Results obtained from an experimental model also showed that the IgM-capture ELISA can detect IgM with 100% sensitivity, 5-42 days post-infection, with the sensitivity decreasing to 12.5% three weeks later (Paweska *et al.* 2003).

During the second bleeding on the affected farm, IgM antibodies could be detected in 2.1% (5/234) of cattle sera. The difference between the results obtained during the two collections, is not statistically significant (*P=0.089*). According to the data above, explaining the presence of IgM and the decreased sensitivity of the IgM-capture ELISA, the presence of IgM antibodies during the second bleeding could be either persisting IgM from the first infection or IgM from a more recent infection during the eight weeks after the first bleeding.

Results from sera collected during the second bleeding showed that there was a statistically significant (*P=0.0057*) increase in IgG positive cattle cases from 13.7% (32/233) to 35% (82/234) on the affected farm. The increase in number of IgG positive cattle can be explained in three ways. Firstly, all IgG positive animals [13.7% (32/233)] from the first bleeding should stay positive, secondly, 7 animals tested only IgM positive during the first bleed should have become IgG positive by the second bleeding and contributed 3% (7/234) to the increase of IgG positive animals; and finally, the remaining increase of about 18.4% (43/234) of IgG positive animals could have been from animals that tested negative for both IgM and IgG during the first bleeding, but sero-converted between the two blood collections. Blood could therefore have been collected before any detectable antibody titre development.

The low number of sheep (1st bleeding 1/73 and 2nd bleeding 1/85 IgM positive; 2/73 IgG positive) that tested positive with the ELISA and all samples that tested negative with the SNT as well as the absence of clinical disease in sheep, indicate that sheep in this study did not get infected. This is in contrasts with published data indicating the high susceptibility of sheep to RVF virus (Findlay 1931; Gerdes 2004) but could be explained that mosquito vectors circulating in the area were more host-specific and had a preference for cattle.

No deaths or clinical disease were reported on the neighbouring farm. In addition, no IgM antibodies could be detected in sera collected on this farm (first bleeding 19 March), suggesting that no active infection had been present at that time. However, the presence of IgG in 5.5% of sera collected (first bleeding, 19 March) on this neighbouring farm in the absence of a vaccination programme, clearly indicated that the virus had been circulating there during February/ March 2008 or before.

The SNT is considered the gold standard serological test for the diagnosis of RVF infections (OIE 2009). In this study one bovine (1/23) sample (spu06/09/117, Appendix 5) that tested weakly IgG positive and three (3/14) samples (spu06/09/294, spu06/09/434 and spu06/09/487, Appendix 5) that tested weakly IgM positive, were negative using the SNT. On the other hand two (2/19) sera tested negative on both ELISA's were weakly positive using the SNT. In experimentally infected and in vaccinated domestic animals the IgM-capture ELISA was more sensitive to use in the early stages of immunological responses than the SNT (Paweska *et al.* 2003).

Another explanation for the lack of agreement found between results obtained from the ELISA's and the SNT could be the interpretation of borderline readings for the ELISA. The specific animal population tested also influence the interpretation of the results obtained by the ELISA's and the manufacturer recommends re-adjustment of threshold values should be considered for each species (Paweska *et al.* 2003).

No viral nucleic acid was detected using PCR in all the selected IgG and IgM positive as well as negative serum samples. The appearance of IgM antibodies three to five days after the onset of infection coincides with the cessation of viraemia (FAO 2003). According to the information that was sent to the OIE by the South African Veterinary Services (OIE Ref: 7083), the outbreak at Bela-Bela started on 1 March 2008. The first bleeding took place 19/20 days later and by this time 56.3% of IgM positive cattle were already IgG positive. These facts are in agreement with the unsuccessful detection of viral nucleic acid by PCR because the viraemia in the animals had already disappeared by the time of sampling.

Although the PCR results were negative, the presence of IgM and RVF positive histopathological and immunochemistry findings in these calves indicated the recent circulation of virus. The outbreak occurred during the end of summer (March) when

infected mosquito vectors should still be present and could therefore be incriminated of transmitting the virus between the two collections.

How the virus was introduced onto this farm remains unclear, however, the possibility of low level virus circulation in animals as described for endemic areas is a possibility (Swanepoel & Coetzer 2004). Although the mechanism of such a circulation is not well understood, reactivation of virus from these endemic foci may occur when vector competent mosquitoes are present. This could also be partially supported by the IgG antibodies detected on the two farms during the first bleeding.

The movement of mosquito vectors by wind over long distances might have introduced the virus to the farm (Sellers *et al.* 1982). This and the presence of a low-lying area with a water stream nearby on the farm that favoured the breeding of mosquitoes may have led to ideal circumstances for an outbreak to occur. The fact that mostly cattle seroconverted suggests a higher host preference of the local population of mosquitoes for cattle rather than sheep.

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APPENDIX 1: ELISA plate layout

1.1. Plate layout: IgM ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	C++	C++	1	2	3	4	5	6	7	8	9	10
B	C++	C++	1	2	3	4	5	6	7	8	9	10
C	C+	C+	11	12	13	14	15	16	17	18	19	20
D	C-	C-	11	12	13	14	15	16	17	18	19	20
E	C++	C++	1	2	3	4	5	6	7	8	9	10
F	C++	C++	1	2	3	4	5	6	7	8	9	10
G	C+	C+	11	12	13	14	15	16	17	18	19	20
H	C-	C-	11	12	13	14	15	16	17	18	19	20

Rows A-D 1-12: RVF virus antigen

Rows E-H 1-12: control antigen

1 to 20 : Test sera

1.2. Plate layout: recN IgG Indirect ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	C++	C++	1	2	3	4	5	6	7	8	9	10
B	C++	C++	1	2	3	4	5	6	7	8	9	10
C	C+	C+	11	12	13	14	15	16	17	18	19	20
D	C+	C+	11	12	13	14	15	16	17	18	19	20
E	C-	C-	21	22	23	24	25	26	27	28	29	30
F	C-	C-	21	22	23	24	25	26	27	28	29	30
G	CC	CC	31	32	33	34	35	36	37	38	39	40
H	CC	CC	31	32	33	34	35	36	37	38	39	40

APPENDIX 2: Examples of ELISA raw data

Plate: Plate 2 anti-RVF virus IgM							Date Created: 2009/03/03 04:00:58 PM				
Raw Data											
1.475	1.632	0.3	0.441	0.211	0.333	0.928	0.25	2.175	0.732	0.305	0.341
1.634	1.635	0.299	0.365	0.172	0.276	0.823	0.215	1.995	0.914	0.279	0.412
0.568	0.475	0.362	0.252	0.313	0.366	0.255	0.201	0.336	0.188	2.461	2.911
0.169	0.204	0.309	0.263	0.253	0.306	0.246	0.149	0.269	0.197	2.441	2.997
0.173	0.152	0.262	0.324	0.135	0.213	0.148	0.17	0.205	0.173	0.254	0.144
0.173	0.154	0.262	0.364	0.14	0.205	0.152	0.176	0.208	0.162	0.249	0.152
0.174	0.158	0.3	0.235	0.289	0.305	0.232	0.125	0.243	0.164	0.158	0.155
0.19	0.157	0.288	0.238	0.263	0.281	0.22	0.154	0.297	0.162	0.186	0.152

Plate: Plate 1 anti-RVF virus IgG							Date Created: 2009/03/04 01:32:11 PM				
Raw Data											
1.987	1.582	0.239	0.342	0.32	0.257	0.174	0.213	0.319	0.242	0.212	0.278
1.727	1.29	0.259	0.275	0.27	0.208	0.132	0.176	0.341	0.222	0.206	0.289
0.639	0.557	1.021	0.233	0.235	0.202	0.257	0.21	0.225	0.182	0.279	0.399
0.166	0.158	0.909	0.193	0.219	0.157	0.197	0.182	0.184	0.17	0.2	0.291
1.837	1.552	0.239	0.179	0.261	0.147	0.26	0.178	0.245	0.383	0.242	0.202
1.741	2.367	0.229	0.165	0.275	0.136	0.243	0.174	0.229	0.346	0.19	0.141
0.587	0.628	0.208	0.13	0.153	0.195	0.142	0.231	0.162	0.214	0.2	0.191
0.124	0.121	0.229	0.152	0.806	0.205	0.165	0.245	0.183	0.232	0.208	0.19

Plate: Plate 2 anti-RVF virus IgG							Date Created: 2009/03/04 01:34:53 PM				
Raw Data											
1.996	1.839	0.217	0.155	0.165	0.235	0.252	0.295	0.246	0.11	0.137	0.137
2.075	1.728	0.187	0.143	0.162	0.216	0.218	0.244	0.237	0.121	0.177	0.124
0.694	0.628	0.161	0.127	0.183	0.192	0.09	0.16	0.168	0.166	0.326	0.166
0.167	0.148	0.174	0.141	0.202	0.225	0.104	0.157	0.175	0.158	0.308	0.164
1.552	1.333	0.155	0.132	0.268	0.165	0.343	0.248	0.135	0.16	0.17	0.158
1.544	1.251	0.14	0.148	0.202	0.149	0.313	0.25	0.141	0.158	0.184	0.14
0.719	0.567	0.161	0.232	0.205	0.162	0.233	0.204	0.253	0.19	0.158	0.184
0.229	0.24	0.141	0.185	0.177	0.18	0.209	0.159	0.196	0.152	0.145	0.197

APPENDIX 3: Example of IgG ELISA PP value calculation and results interpretation

Reference number	OD1	OD2	C++ Average: (1.727+1.582)/2 = 1.6545		Interpretation
			OD Average	PP value	
C++	1.987	1.582	OD1 + OD2 / 2	OD Average x 100 / C++ Average	
C++	1.727	1.29			
C+	0.639	0.557	0.598	36.14385	
C-	0.166	0.158	0.162	9.791478	
CC	0.124	0.121	0.1225	7.40405	
spu066/09/001	0.239	0.259	0.249	15.04986	Negative
spu066/09/002	0.342	0.275	0.3085	18.64612	Negative
spu066/09/003	0.32	0.27	0.295	17.83016	Negative
spu066/09/004	0.257	0.208	0.2325	14.05258	Negative
spu066/09/005	0.174	0.132	0.153	9.247507	Negative
spu066/09/006	0.213	0.176	0.1945	11.75582	Negative
spu066/09/007	0.319	0.341	0.33	19.9456	Negative
spu066/09/008	0.242	0.222	0.232	14.02236	Negative
spu066/09/009	0.212	0.206	0.209	12.63222	Negative
spu066/09/010	0.278	0.289	0.2835	17.13509	Negative
spu066/09/011	**1.021**	**0.909**	**0.965**	**58.32578**	**Positive**
spu066/09/012	0.233	0.193	0.213	12.87398	Negative
spu066/09/013	0.235	0.219	0.227	13.72016	Negative
spu066/09/014	0.202	0.157	0.1795	10.8492	Negative
spu066/09/015	0.257	0.197	0.227	13.72016	Negative
spu066/09/016	0.21	0.182	0.196	11.84648	Negative
spu066/09/017	0.225	0.184	0.2045	12.36023	Negative
spu066/09/018	0.182	0.17	0.176	10.63765	Negative
spu066/09/019	0.279	0.2	0.2395	14.47567	Negative
spu066/09/020	0.399	0.291	0.345	20.85222	Negative
spu066/09/021	0.239	0.229	0.234	14.14325	Negative
spu066/09/022	0.179	0.165	0.172	10.39589	Negative
spu066/09/023	0.261	0.275	0.268	16.19825	Negative
spu066/09/024	0.147	0.136	0.1415	8.552433	Negative
spu066/09/025	0.26	0.243	0.2515	15.20097	Negative
spu066/09/026	0.178	0.174	0.176	10.63765	Negative
spu066/09/027	0.245	0.229	0.237	14.32457	Negative
spu066/09/028	0.383	0.346	0.3645	22.03083	Negative
spu066/09/029	0.242	0.19	0.216	13.0553	Negative
spu066/09/030	0.202	0.141	0.1715	10.36567	Negative
spu066/09/031	0.208	0.229	0.2185	13.20641	Negative
spu066/09/032	0.13	0.152	0.141	8.522212	Negative
spu066/09/033	0.153	0.806	0.4795	28.98157	Negative
spu066/09/034	0.195	0.205	0.2	12.08824	Negative
spu066/09/035	0.142	0.165	0.1535	9.277727	Negative
spu066/09/036	0.231	0.245	0.238	14.38501	Negative
spu066/09/037	0.162	0.183	0.1725	10.42611	Negative
spu066/09/038	0.214	0.232	0.223	13.47839	Negative
spu066/09/039	0.2	0.208	0.204	12.33001	Negative
spu066/09/040	0.191	0.19	0.1905	11.51405	Negative

APPENDIX 4: Example of IgM ELISA PP value calculation and results interpretation

Reference number	OD1	OD2	C++ Average: (1.461+1.48)/2 = 1.4705		Interpretation
			OD Average	PP value	
C++	1.302	1.48	OD1 + OD2 / 2	OD Average x 100 / C++ Average	
C++	1.461	1.481			
C+	0.394	0.317	0.3555	24.17545053	
C-	1.302	0.047	0.013	0.884053043	
spu066/09/222	0.038	0.037	0.0375	2.550153009	Negative
spu066/09/230	0.117	0.001	0.059	4.012240734	Negative
spu066/09/235	0.076	0.032	0.054	3.672220333	Negative
spu066/09/236	0.12	0.071	0.0955	6.494389663	Negative
spu066/09/239	**0.78**	**0.671**	**0.7255**	**49.33696022**	**Positive**
spu066/09/245	0.08	0.039	0.0595	4.046242775	Negative
spu066/09/254	**1.97**	**1.787**	**1.8785**	**127.7456647**	**Positive**
spu066/09/259	**0.555**	**0.746**	**0.6505**	**44.2366542**	**Positive**
spu066/09/290	0.051	0.03	0.0405	2.75416525	Negative
spu066/09/294	**0.197**	**0.26**	**0.2285**	**15.53893234**	**Positive**
spu066/09/300	0.062	0.021	0.0415	2.82216933	Negative
spu066/09/304	0.017	0.025	0.021	1.428085685	Negative
spu066/09/310	0.024	-0.01	0.007	0.476028562	Negative
spu066/09/311	0.061	0.025	0.043	2.924175451	Negative
spu066/09/322	0.023	0.026	0.0245	1.666099966	Negative
spu066/09/329	0.076	-0.005	0.0355	2.414144849	Negative
spu066/09/336	0.093	-0.028	0.0325	2.210132608	Negative
spu066/09/359	0.022	0.036	0.029	1.972118327	Negative
spu066/09/374	**2.303**	**2.255**	**2.279**	**154.9812989**	**Positive**
spu066/09/377	**2.756**	**2.848**	**2.802**	**190.5474328**	**Positive**

APPENDIX 5: SNT results of selected samples

Sample Number	IgG ELISA		SNT Results
	PP	Interpr.	
spu066/09/183	163.60	Positive	1:640
spu066/09/680	171.65	Positive	>1:640
spu066/09/671	191.13	Positive	>1:640
spu066/09/90	150.19	Positive	>1:640
spu066/09/125	119.22	Positive	1: 320
spu066/09/94	111.15	Positive	1: 320
spu066/09/140	92.31	Positive	>1:160
spu066/09/160	84.91	Positive	>1:320
spu066/09/154	81.34	Positive	>1:320
spu066/09/114	76.92	Positive	>1:160
spu066/09/128	76.40	Positive	1:320
spu066/09/107	74.00	Positive	1:320
spu066/09/228	44.55	Positive	1:160
spu066/09/242	57.18	Positive	1:40
spu066/09/269	32.71	Positive	1:20
spu066/09/166	36.72	Positive	>1:80
spu066/09/223	36.51	Positive	1:20
spu066/09/219	37.49	Positive	1:80
spu066/09/181	36.85	Positive	1:20
spu066/09/186	38.42	Positive	1:20
spu066/09/159	39.12	Positive	1:40
spu066/09/489	38.98	Positive	1:320
spu066/09/117	30.45	Positive	Negative
spu066/09/412	28.76	Negative	Negative
spu066/09/375	27.18	Negative	Negative
spu066/09/605	27.35	Negative	Negative
spu066/09/606	28.38	Negative	Negative
spu066/09/368	26.84	Negative	Negative
spu066/09/621	26.22	Negative	Negative
spu066/09/629	26.83	Negative	Negative
spu066/09/460	4.64	Negative	Negative
spu066/09/167	4.48	Negative	Negative
spu066/09/230	19.98	Negative	1:20
spu066/09/236	25.82	Negative	1:10

Sample Number	IgM ELISA		SNT Results
	PP	Interpr.	
spu066/09/632	287.30	Positive	1:320
spu066/09/254	127.75	Positive	>1:640
spu066/09/374	154.98	Positive	1:160
spu066/09/377	190.55	Positive	1:160
spu066/09/493	170.27	Positive	>1:640
spu066/09/494	155.07	Positive	>1:640
spu066/09/495	166.81	Positive	>1:640
spu066/09/497	133.37	Positive	1:320
spu066/09/239	49.34	Positive	1:20
spu066/09/498	168.48	Positive	>1:640
spu066/09/499	126.51	Positive	>1:640
spu066/09/294	15.54	**Positive**	Negative
spu066/09/487	25.16	**Positive**	Negative
spu066/09/434	14.36	**Positive**	Negative
spu066/09/123	1.85	Negative	Negative
spu066/09/134	1.76	Negative	Negative
spu066/09/587	7.16	Negative	Negative
spu066/09/591	5.82	Negative	Negative
spu066/09/480	0.60	Negative	Negative
spu066/09/424	0.87	Negative	Negative
spu066/09/427	-0.24	Negative	Negative
spu066/09/169	-0.09	Negative	Negative
spu066/09/230	19.98	Negative	1:20
spu066/09/236	25.82	Negative	1:10