Monitoring of humoral immune responses against Marburg virus and evaluation of their role in protection against re-challenge in naturally immune *Rousettus aegyptiacus* fruit bats

by

Nadia Storm

Supervisor: Prof Janusz Paweska

Co-supervisors: Dr Petrus Jansen van Vuren, Prof Wanda Markotter

A thesis submitted in partial fulfilment of the requirements for the degree

Philosophiae Doctor (Microbiology)

in the Department of Biochemistry, Genetics and Microbiology

Faculty of Natural and Agricultural Sciences

University of Pretoria

Pretoria

South Africa

2018

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

I, Nadia Storm, declare that the thesis hereby submitted to the University of Pretoria for the degree PhD (Microbiology) and the work contained therein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree.

Signed: _____this 15th day of November 2018.

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to the following:

My supervisors

I would like to thank each one of you for taking me on as a student and guiding me throughout this project. Your time, knowledge, patience, support and advice were invaluable in the completion of this thesis, and in my professional development as a young scientist.

Prof Janusz Paweska – thank you for the numerous opportunities you have afforded me to follow my passion and develop my research career, including allowing me to participate in the Ebola outbreak response mission to Sierra Leone in 2014. Thank you for your guidance and expert opinion, for helping me steer this project into better directions when other avenues failed, and for allowing me to use the BSL-4 facility and other CEZPD infrastructure.

Dr Petrus Jansen van Vuren – thank you for spending several hours training me in techniques that were crucial to the successful outcome of this project, for chaperoning me in my early BSL-4 days and for always having an open door and a sympathetic ear.

Prof Wanda Markotter – thank you for your critical inputs throughout my project, for your effort and commitment towards your students, for teaching me so much about bats, and for instilling in me a deep interest in these animals and the viruses that are associated with them.

From the Research Centre for Zoonosis Control, Hokkaido University, Sapporo, Japan

Prof Ayato Takada, Dr Junki Maruyama and Ms Hiroko Miyamoto – thank you for teaching me the techniques to clone, express and purify the recombinant proteins used in the serological assays of this study, and for gifting me the plasmids required to do so. Thank you to all other staff and students in the department for making me feel at home in Japan.

From the Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD), National Institute for Communicable Diseases of the National Health Laboratory Service (NICD-NHLS)

Mr Günther Meier, Mr Zibusiso Masuku and Mr Ronny Sebaka – thank you for assisting me with the technical aspects of this project.

Dr Jacqueline Weyer, Dr Naazneen Moolla, Mrs Antoinette Grobbelaar, Ms Chantel Le Roux, Dr Gary Robertson, Mrs Pat Leman and Mr Alan Kemp – thank you for your advice, words of encouragement and ongoing support.

Mr Justice Kgatitsoe and Mrs Busi Mogodi – thank you for your help with the collection of bat specimens and animal husbandry.

Ms Nondumiso Mpuhlu – thank you for providing administrative assistance with this project.

From the University of Pretoria

My fellow students, Dr Marike Geldenhuys, Ms Marinda Mortlock, Dr Jessica Coertse and Dr Low de Vries – thank you for assisting with the collection of bat specimens for this project, and for your friendship and encouragement.

For funding

The National Research Foundation - grant 86228. The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the author and are not necessarily to be attributed to the NRF.

The Poliomyelitis Research Foundation - grants 13/11 (Dr P Jansen van Vuren) and 13/54 (N Storm).

The University of Pretoria - postgraduate research bursary (PhD) and study abroad bursary.

Prof William James and the judging panel of the James S Porterfield Prize in International Virology – thank you for awarding me the prize in 2014, which allowed me to complete an essential part of my project in Japan.

The Defense Threat Reduction Agency of the United States of America, and Whitehead Scientific – thank you for providing funding for attending and presenting part of my research at the One Health EcoHealth Conference in 2016.

My family

My parents, Leoni and Kobie - thank you for loving, encouraging and supporting me throughout this degree and my life. Thank you for giving me the strength to keep going, and for reminding me to never give up on my dreams.

My husband, Paul - thank you for believing in me when I didn't believe in myself, for words of encouragement, for consoling me when experiments failed and for celebrating with me when I had even little breakthroughs. I could not have seen this project through without your tireless love and support.

SUMMARY

Monitoring of humoral immune responses against Marburg virus and evaluation of their role in protection against re-challenge in naturally immune *Rousettus aegyptiacus* fruit bats

by

Nadia Storm

Supervisor: Prof JT Paweska

Head of Department
Centre for Emerging Zoonotic and Parasitic Diseases
National Institute for Communicable Diseases of the National Health Laboratory Service

Extraordinary Professor
Department of Medical Virology
School of Medicine
Faculty of Health Sciences
University of Pretoria

Co-supervisors: Dr P Jansen van Vuren

Medical Scientist
Centre for Emerging Zoonotic and Parasitic Diseases
National Institute for Communicable Diseases of the National Health Laboratory Service

Extraordinary Lecturer
Department of Medical Virology
School of Medicine
Faculty of Health Sciences
University of Pretoria

Prof W Markotter

Head of Department
Centre for Viral Zoonoses
Department of Medical Virology
Faculty of Health Sciences
University of Pretoria

For the degree PhD (Microbiology)

Marburg virus (MARV) is a zoonotic virus of significant potential public health concern in Africa. Together with Ebola virus (EBOV), MARV belongs to the family *Filoviridae* and causes a life-threatening haemorrhagic disease in humans and non-human primates. The occurrence of large outbreaks of MARV disease (MVD) within the past two decades, as well as the devastating EBOV outbreaks in West and Central Africa, indicates that filoviruses are a much more significant public health threat than previously anticipated and can emerge at any time without warning. These unprecedented outbreaks have emphasised the need for surveillance in reservoir host populations and for safe and reliable surveillance tools and diagnostic tests that may easily be performed in both laboratory and field settings. Egyptian rousette bats (ERB; *Rousettus aegyptiacus*) are reservoir hosts for MARV, and there is a need for understanding the dynamics of immune responses of these animals to MARV infection. This knowledge can assist in predicting periods of increased transmission within bat colonies and in turn, potential spillover events into human and other animal populations.

In this thesis, the development of indirect enzyme-linked immunosorbent assays (I-ELISA) for the detection of specific anti-MARV immunoglobulin G (IgG) in bat sera is described. The I-ELISAs, based on two recombinant MARV protein antigens (nucleoprotein and glycoprotein), can be used without the need for high biocontainment facilities. Both I-ELISAs were found to be robust and repeatable, with good sensitivity and specificity. Applying the I-ELISAs in detecting IgG antibodies to MARV in sera collected from both wild-caught and experimentally infected bats indicated that the assays are suitable methods for MARV serosurveillance, with

the MARV GP-based I-ELISA demonstrating higher diagnostic performance compared to the MARV NP-based assay.

Based on this knowledge, the MARV GP I-ELISA was applied in monitoring and characterising the antibody responses of ERBs to MARV. Maternal antibodies to MARV were detected in juvenile bats up to approximately five months after birth. In bats experimentally infected with MARV, antibodies against the virus remained detectable in the majority of bats at 110 days post-infection. Furthermore, antibodies to MARV remained detectable in 84% of naturally exposed bats at least 11 months after capture, suggesting that bats develop long-term humoral immunity in response to active infection with MARV. To test whether pre-existing immunity in bats is protective against reinfection, 15 ERBs with differing levels of MARV-specific IgG antibodies were inoculated with the Watsa isolate of the virus. Levels of anti-MARV IgG antibodies increased swiftly from day 5 post inoculation. Viraemia was detected in 73% of reinfected bats, and the challenge virus was isolated from the serum of one reinfected bat. Viral ribonucleic acid was detected in the spleen (73% of bats), liver (47%) and lung (7%) at different days post inoculation. These results suggest that primary infection of ERBs with MARV does not induce sterilising humoral immunity; however, re-inoculation of previously infected bats produced only localised infection, with an absence of the virus in tissues potentially involved in viral transmission. Reinfection of previously infected bats is therefore not likely to be a key factor driving MARV maintenance in nature.

The establishment of in-house capacity for the production of recombinant I-ELISA antigens as described in this thesis will assist in the biosurveillance programme in South Africa aimed at monitoring the presence and distribution of MARV infection in local bat populations. The assays based on these antigens will also assist in monitoring the immune status of reservoir host populations, predicting potential spillover events, implementing risk reduction strategies and improving virus-host modelling studies. These tools will further contribute to the characterisation of the antibody responses of ERBs to MARV, which may ultimately assist in elucidating the mechanisms by which bats are able to combat clinical MARV disease.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

µ	Micro
ABTS	2,2'-Azino di-ethyl-benzothiazoline-sulfonic acid substrate
BDBV	Bundibugyo virus
bp	Base pair
BSL-4	Biosafety level four
C++	Strong positive control serum
C-	Negative control serum
C_b	Bias correction factor
CCC	Lin's concordance correlation coefficient
CCL2	C-C motif chemokine ligand 2
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CDC	Centres for Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CEZPD	Centre for Emerging Zoonotic and Parasitic Diseases
cm	Centimetre
CO_2	Carbon dioxide
CPE	Cytopathic effect
CsCl	Cesium chloride
Ct	Cycle threshold
CV	Coefficient of variation
DMEM	Dulbecco's modified Eagle medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
DRC	Democratic Republic of the Congo
DSe	Diagnostic sensitivity
DSp	Diagnostic specificity
DTT	Dithiothreitol
EBOV	Ebola virus

E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimal essential medium
ERB	Egyptian rousette bat
FBS	Foetal bovine serum
g	Gravitational force
g	Gram
GP	Glycoprotein
HEK 293T	Human embryonic kidney 293 cells with SV40 large T-antigen
HRP	Horseradish peroxidase
Hz	Hertz
I-ELISA	Indirect enzyme-linked immunosorbent assay
IF	Immunofluorescent assay
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JAK	Janus kinase
KCl	Potassium chloride
kDa	Kilodalton
LB	Luria Bertani
LCL	Lower control limit
MARV	Marburg virus
MCS	Multiple cloning site
MEM	Minimum essential medium
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MHK	Michael Hogan Kidney
ml	Millilitre
mM	Millimolar
M-MLV	Moloney murine leukaemia virus
m/v	Mass per volume
MVD	Marburg virus disease

NHLS	National Health Laboratory Service
NHP	Non-human primate
NICD	National Institute for Communicable Diseases
Ni-NTA	Nickel nitrilotriacetic acid
NK	Natural killer
NLR	Nonobese diabetic-like receptors
nm	Nanometer
NP	Nucleoprotein
NPV	Negative predictive value
OD	Optical density
OIE	World Organisation for Animal Health
PAMP	Pathogen-associated molecular pattern
PBS-T	Phosphate buffered saline containing Tween-20
p.i.	Post inoculation
PP	Percentage positivity
PPV	Positive predictive value
PRNT	Plaque reduction neutralisation test
PRR	Pathogen recognition receptors
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RAVV	Ravn virus
r	Pearson's correlation coefficient
RESTV	Reston virus
RLR	Rig-like receptor
RNA	Ribonucleic acid
rpm	Revolutions per minute
r_s	Spearman's rank-order coefficient
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOC	Super optimal broth with catabolite repression
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
SUDV	Sudan virus

TAFV	Taï Forest virus
TBS	Tris-buffered saline
$TCID_{50}$	50% tissue culture infective dose
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Tris-HCl	Tris hydrochloride
UCL	Upper control limit
USA	United States of America
V	Volt
VHF	Viral haemorrhagic fever
VLP	Virus-like particle
VNT	Virus neutralisation test
WHO	World Health Organisation
w/v	Weight per volume

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

LIST OF TABLES

Chapter 1

Chapter 2

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction and thesis layout

Marburg virus (MARV) is a member of the family *Filoviridae* and causes life-threatening haemorrhagic fever in humans and non-human primates (NHP) (Feldmann *et al*., 2013). Up until 2018, 14 outbreaks of MARV disease (MVD) have been recorded, and several of these have been associated with entry into caves or mines, or contact with bats (Conrad *et al*., 1978; Smith *et al*., 1982; Johnson *et al*., 1996; Bausch *et al*., 2006; Centres for Disease Control and Prevention, 2009; Timen *et al*., 2009; Adjemian *et al*., 2011). The majority of MVD outbreaks have been reported from central, East and southwest Africa (Smith *et al*., 1982; Johnson *et al*., 1996; Bausch *et al*., 2006; Towner *et al*., 2006; World Health Organisation, 2007; World Health Organisation, 2012; World Health Organisation, 2014; World Health Organisation, 2017). However, MARV is also of global significance due to the possibility of introduction to non-endemic countries through animal importation and travel, and the potential for the use of the virus as a biological terrorism agent (Groseth *et al*., 2009).

A large-scale and deadly outbreak of MVD in northern Angola in 2004 - 2005 (Towner *et al*., 2006), followed a decade later by a devastating Ebola virus (EBOV) disease outbreak in West Africa (Baize *et al*., 2014), demonstrated that filoviruses may emerge in unexpected locations without warning, and may be a more significant threat to global health than previously thought. For this reason, surveillance in reservoir hosts is crucial to predict or prevent imminent spillover of MARV into surrounding human and animal populations. In addition, the rapid and accurate diagnosis of MVD is vital in containing and reducing the impact of outbreaks once spillover has occurred.

The high lethality of MARV, coupled with the unavailability of suitable vaccines or therapeutics necessitates that the virus be handled in maximum biosafety laboratories (Brauburger *et al*., 2012). This requirement has hampered the development of safe and reliable assays for MARV diagnosis and surveillance and, together with the unpredictable nature of MVD outbreaks, has made the virus very difficult to study. Consequently, many gaps in

knowledge still exist regarding virulence factors, host-pathogen relationships, host immune responses and natural MARV transmission and maintenance mechanisms.

Bats, specifically Egyptian rousette bats (ERB), are reservoir hosts for MARV (Swanepoel *et al*., 2007; Towner *et al*., 2007; Towner *et al*., 2009; Paweska *et al*., 2012). Bat immunity, in general, is poorly understood, mainly because of the unavailability of bat-specific reagents, immunoassays and cell lines required to study bat immune mechanisms (Schountz, 2014; Baker & Zhou, 2015; Schountz *et al*., 2017; Banerjee *et al*., 2018). As a result, little is known about the immune responses of ERBs to MARV infection. The immune dynamics of bats may play significant roles in the preservation of MARV in nature (Amman *et al*., 2012; Plowright *et al*., 2016). The loss of maternal antibodies in juvenile ERBs is currently thought to be the primary driver of MARV transmission within bat populations (Amman *et al*., 2012; Paweska *et al*., 2018), but information on the duration of maternal immunity in ERBs and when these bats become susceptible to infection is limited. The reinfection of previously exposed bats might play an additional role in the natural maintenance of the virus (Schuh *et al*., 2017a), as it is unclear whether antibodies offer life-long protection to bats against MVD.

The research described in this thesis focused on addressing the need for improved tools to diagnose MVD and perform surveillance for MARV in reservoir host bat populations. In addition, the research aimed to investigate the role of antibody responses of ERBs to MARV in viral maintenance and transmission. This thesis is organised into four chapters. Chapter 1 serves as a review of relevant literature, addressing aspects of MARV morphology, epidemiology, virus hosts, immune evasion strategies, host immune responses and availability of assays for MARV diagnosis and surveillance. The development and evaluation of tools for the detection of antibodies to MARV in bat sera is described in chapter 2. The application of these tools in the monitoring and characterisation of the active and passive antibody responses of ERBs to MARV, and the role of antibodies in the protection of these bats against reinfection is described in chapter 3. Finally, concluding remarks and future directions are presented in chapter 4.

1.2 History and taxonomy of Marburg virus

Marburg virus disease was first reported in August 1967 in Germany and Serbia (formerly Yugoslavia), when laboratory technicians became infected with a novel agent while handling blood and tissue specimens of African green monkeys (*Chlorocebus aethiops*) which were shipped from Uganda to Europe (Martini, 1969). Thirty-one of the laboratory workers developed a severe haemorrhagic disease, of which seven did not survive (Martini, 1969). As a result, three shipments of monkeys were euthanised, and the outbreak was rapidly contained. During this period, MARV was isolated and characterised for the first time, and named after the city in which most cases occurred (Siegert *et al*., 1968a). Marburg virus disease was not reported again until 1975, when an Australian man acquired the infection while travelling through Zimbabwe to Johannesburg, South Africa, and spread the virus to a travel companion and a nurse (Gear *et al*., 1975). The man had reportedly slept in a room inhabited by bats and had possibly visited the Chinhoyi cave in Zimbabwe shortly before falling ill (Conrad *et al*., 1978), but the exact source of infection was never determined. The man succumbed to MVD, but fortunately, both his travel companion and the nurse survived (Gear *et al*., 1975).

A year later, Sudan virus (SUDV) was discovered in Nzara, Sudan, when an outbreak of haemorrhagic fever resembling MVD originated among cotton factory workers in the area (World Health Organisation, 1978a). Around the same time, cases of haemorrhagic fever were described in the northern parts of the Democratic Republic of the Congo (DRC) (formerly known as Zaire). The outbreak was initiated when the index case received a chloroquine injection for suspected malaria at the Yambuku Mission Hospital and afterwards presented with haemorrhagic fever symptoms (World Health Organisation, 1978b). Nursing staff were supplied with only five needles and syringes each day, and because the needles were not sterilised between patients, subsequent patients receiving these injections also acquired an unknown haemorrhagic disease and spread the infection to close contacts and hospital staff members (World Health Organisation, 1978b). The unidentified causative agent was soon isolated and named EBOV, after which marburgviruses and ebolaviruses were classified together in the family *Filoviridae*.

As of 2018, the family *Filoviridae* is one of eight families of viruses assigned to the order *Mononegavirales* and consists of the genera *Marburgvirus*, *Ebolavirus* and *Cuevavirus* (Amarasinghe *et al*., 2018). The genus *Marburgvirus* contains a single species, *Marburg marburgvirus* (formerly *Lake Victoria marburgvirus*), which includes two marburgviruses, named Ravn virus (RAVV) and MARV (Amarasinghe *et al*., 2018).

1.3 Morphology, genomic structure and genetic diversity of Marburg virus

Marburg virus particles are filamentous and pleomorphic, appearing under an electron microscope as rod-, ring-, six- or crook-shaped structures (Bharat *et al*., 2011). The virus has a uniform diameter of 90 nm and is approximately 900 nm in length (Bharat *et al*., 2011); however, an earlier publication has indicated that MARV particles may reach lengths of up to 14 000 nm in infected cell culture (Geisbert & Jahrling, 1995). Marburg virions consist of a helical nucleocapsid enclosed by a lipid envelope (Sanchez *et al*., 1992). The MARV genome is approximately 19 kilobases in length and consists of non-segmented, negative-sense, single-stranded ribonucleic acid (RNA) rich in uridine and adenosine residues (Feldmann *et al*., 2013). The genome contains seven monocistronic genes (Figure 1.1) which may either be separated by intergenic regions of up to 97 nucleotides, or the upstream gene transcription stop signals may overlap with the downstream gene transcription start signals with five highly conserved nucleotides, a characteristic which is unique to the order *Mononegavirales* (reviewed in Brauburger *et al*., 2012).

To date, at least nine genetically distinct strains of MARV have been identified, including Popp (Bukreyev *et al*., 1995), Ci67 (Siegert *et al*., 1968b), Ozolin (Gear *et al*., 1975), the prototype Musoke (Smith *et al*., 1982), Ravn (Johnson *et al*., 1996), Angola (Towner *et al*., 2006) and strains from the initial MVD outbreak in Germany and Serbia. Based on a comparative analysis of the glycoprotein (GP) and viral structural protein 35 (VP35) of MARV, these strains are divided into two separate lineages within the *Marburg marburgvirus* species, with the RAVV strain representing its own lineage (Sanchez *et al*., 1998).

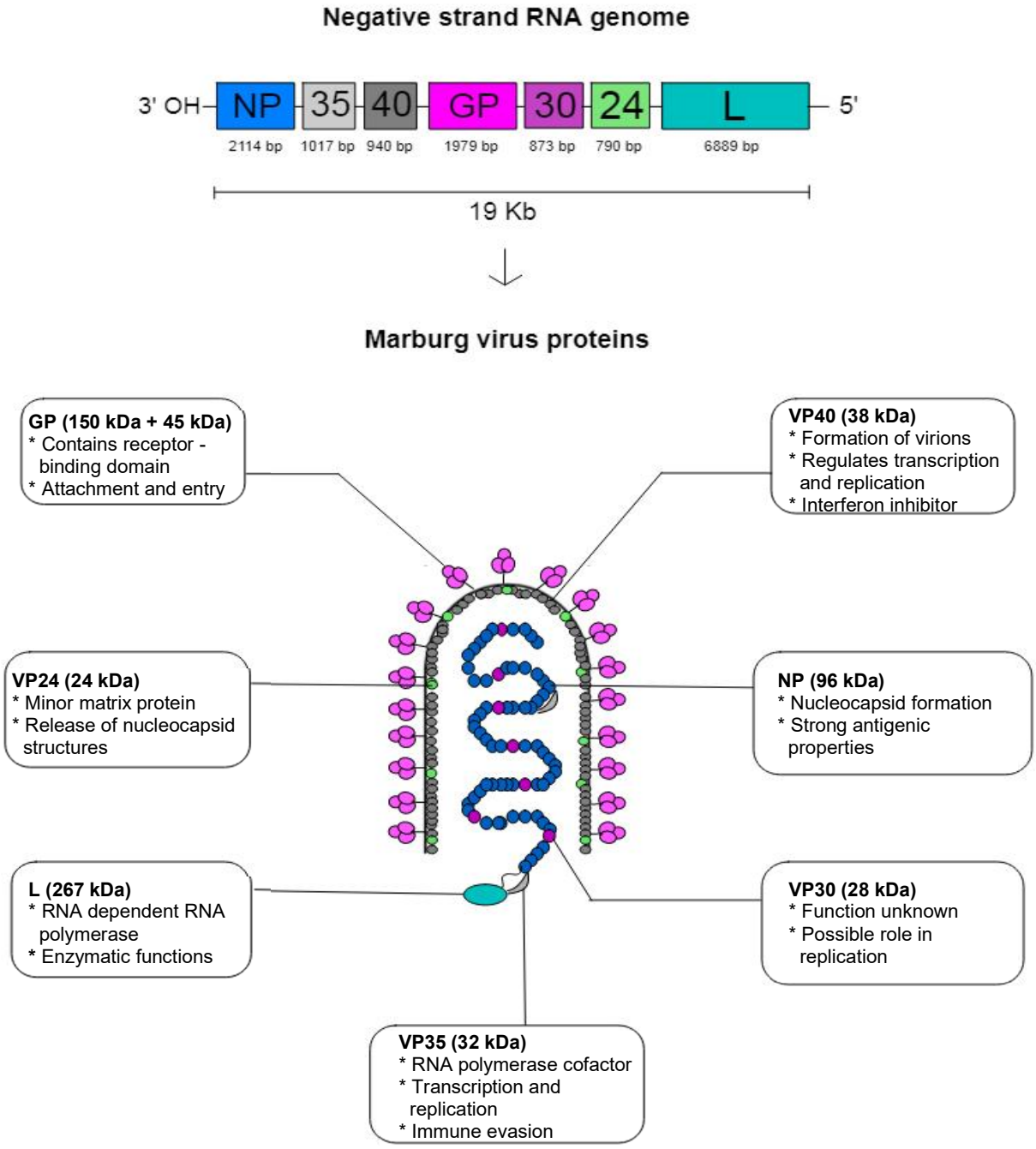


Figure 1.1: Genomic structure and gene products of Marburg virus. Abbreviations: OH – hydroxide, NP – nucleoprotein, VP – viral structural protein, GP – glycoprotein, L – polymerase, bp – base pair, kDa - kilodalton. Genes and gene products are colour coded and sizes are indicated.

Ravn virus and other MARV genomes differ by approximately 20% at the nucleotide level, but a high number of amino acids remain conserved in all but the GP gene of these strains (maximum amino acid difference of 12.3% versus 23.5% for the GP gene) (Towner *et al*., 2006; Carroll *et al*., 2013). The genomes of MARV isolates originating from Angola, southwest Africa, differ from East African isolates by approximately seven percent at the nucleotide level (Towner *et al*., 2006). In MVD outbreaks where a single introduction into the human population from a reservoir host most likely occurred, nucleotide differences of less than one percent at the genomic level existed between isolates from different clinical cases (Towner *et al*., 2006). In contrast, outbreaks associated with numerous independent introductions of MARV into the human population were associated with highly divergent clinical isolates, with a nucleotide difference of up to 21% at the genomic level (Towner *et al*., 2006).

1.3.1 Marburg virus proteins and functions

The MARV genes code for seven structural proteins, including a nucleoprotein (NP), viral structural proteins 24 (VP24), 30 (VP30), 35 and 40 (VP40), a GP and an RNA-dependent RNA polymerase (L) (Peters, 2005; reviewed in Kajihara & Takada, 2015) (Figure 1.1). The most conserved proteins between different MARV strains include VP40, NP, VP24 and VP35, while the most divergent protein is the GP (Towner *et al*., 2006). The NP, VP30, VP35, and L form the ribonucleoprotein complex, while GP, VP24 and VP40 are associated with the lipid envelope (Peters, 2005).

1.3.1.1 Nucleoprotein

The first MARV gene encodes for NP (96 kilodalton (kDa)), which surrounds the viral RNA, protects the genome from nucleases and drives nucleocapsid formation (Mavrakis *et al*., 2002). The NP consists of a hydrophobic N-terminal half and an acidic, hydrophilic C-terminal half (Sanchez *et al*., 1992). The MARV NP is highly conserved, with a maximum amino acid difference of 5.9% between different MARV strains (Towner *et al*., 2006). Although an amino acid difference of up to almost 70% exists between marburg- and ebolavirus NPs, filoviral NPs show strong amino acid sequence homology in the 400 residues at the N-terminus of the protein (Sanchez *et al*., 1992). This region likely forms structures that are functionally relevant to the

Filoviridae family, such as RNA binding. The NP of MARV exhibits strong antigenic properties, making it an ideal target for antigen detection tests (Saijo *et al*., 2001a; Niikura *et al*., 2003; Changula *et al*., 2013). The majority of epitopes for anti-NP antibodies have been identified in the C-terminal half of this protein (Saijo *et al*., 2001; Saijo *et al*., 2005; Changula *et al*., 2013), at least one of which is conserved between all filovirus species (Ali & Islam, 2015).

1.3.1.2 Viral structural protein 35

The VP35 gene of MARV varies by 5.5% at the amino acid level between different strains (Towner *et al*., 2006). Viral structural protein 35 (32 kDa) is a cofactor for RNA polymerase and is essential for MARV transcription and replication (Mühlberger *et al*., 1998). Additionally, VP35 plays a crucial role in immune evasion by acting as an interferon (IFN) antagonist, suppressing dendritic cell maturation (Yen *et al*., 2014) and preventing the launch of the innate immune response (reviewed in Audet & Kobinger, 2015).

1.3.1.3 Viral structural protein 40

The third gene of MARV encodes for the membrane-associated protein VP40 (38 kDa), which is the most abundant protein in the virion (Feldmann & Klenk, 1996). The VP40 gene is the most conserved between different MARV strains (1.7% maximum variation at the amino acid level) (Towner *et al*., 2006) and shows little tolerance for amino acid substitutions. The VP40 of MARV assembles on the inner plasma membrane of human cells, where budding of the virus is regulated and virus-like particles (VLPs) may be produced without the presence of other viral proteins (Noda *et al*., 2002). Mutations in a loop region of the N-terminal domain of VP40 have been shown to reduce plasma membrane localisation of the protein, as well as the release of VLPs from cells (Adu-Gyamfi *et al*., 2014). Viral structural protein 40 is essential for the formation of virions and regulates transcription and replication (Wenigenrath *et al*., 2010). The protein also plays a role in counteracting the human innate immune response by inhibiting IFN signalling (Valmas *et al*., 2010; Valmas & Basler, 2011). In experimentally rodent-adapted variants of MARV, the majority of amino acid changes seem to occur in the VP40 gene (Lofts

et al., 2007; Warfield *et al*., 2009; Lofts *et al*., 2011), suggesting a potential role for the protein in host tropism.

1.3.1.4 Glycoprotein

Marburg virus possesses a single surface protein, GP, which inserts into a host-derived membrane to form trimeric spikes (Feldmann *et al*., 1991; Mittler *et al*., 2011). The GP is the least conserved filoviral protein, with a maximum amino acid variation of 23.5% between different MARV strains (Towner *et al*., 2006) and 72% between different filovirus species (Sanchez *et al*., 1993; Sanchez *et al*., 1998). This high variability is likely due to selective pressure for non-synonymous changes brought about by the immune responses of natural reservoir hosts (Towner *et al*., 2006). Marburg virus GP plays essential roles in virus attachment and entry through association with Niemann-Pick C1 receptors in host cells (Cote *et al*., 2011; Mittler *et al*., 2011), and therefore also in host tropism (Manicassamy *et al*., 2007). Moreover, the GP plays a significant role in viral pathogenesis and has been shown to be the cause of cytopathic effect (CPE) in cell lines (Simmons *et al*., 2002). In the host cell, GP is synthesised as a polypeptide which undergoes glycosylation in the endoplasmic reticulum (Jeffers *et al*., 2002), followed by furin cleavage into two covalently linked subunits (GP_1 (150 kDa) and GP_2 (45 kDa)) in the Golgi apparatus (Volchkov *et al*., 1998). The GP_1 subunit of MARV contains the receptor binding domain (Yaddanapudi *et al*., 2006), and binding of GP_1 to CD209 receptors in dendritic cells, and CLEC4M receptors on the endothelial cells of liver and lymph node sinusoids, enables infection of macrophages (Shimojima *et al*., 2006). The GP_2 subunit is membrane-spanning and contains a putative immunosuppressive domain which may bring about lymphocyte death and repress cytokine responses (Yaddanapudi *et al*., 2006). Additionally, GP_2 may facilitate fusion of the viral and target cell or endosomal membranes, allowing penetration of the virus into the host cell cytoplasm (Shimojima *et al*., 2006).

The majority of epitopes for antibodies are located on the GP of filoviruses (Hevey *et al*., 2003; Fusco *et al*., 2015), and this protein is currently the only known target of neutralising antibodies against MARV (Takada *et al*., 2007a; Takada *et al*., 2007b; Bale *et al*., 2012b). For this reason, this protein is an ideal antigen for use in MARV serological assays and vaccines.

1.3.1.5 Viral structural protein 30

The VP30 gene varies by approximately 10% at the amino acid level between different MARV strains (Towner *et al*., 2006). The function of VP30 (28 kDa) of MARV is poorly understood. In EBOV, VP30 has been shown to play an essential role in controlling transcription initiation (Weik *et al*., 2002; Biedenkopf *et al*., 2016). However, the same has not yet been proven for MARV. Enterlein and colleagues (2006) suggested that VP30 may play an important role in viral replication based on the unsuccessful rescue of MARV in the absence of this protein.

1.3.1.6 Viral structural protein 24

The VP24 is a minor matrix protein of approximately 24 kDa and differs by 4.4% between different MARV strains at the amino acid level (Towner *et al*., 2006). Limited studies have been conducted on the VP24 of MARV. The protein is unique to the family *Filoviridae* and has been suggested to be important in the formation and release of nucleocapsid structures (Noda *et al*., 2007) and viral particles during infection (Bamberg *et al*., 2005). In EBOV, VP24 acts as a type I IFN antagonist (Reid *et al*., 2006), and mutations in this protein allow the virus to adapt to guinea pigs (Volchkov *et al*., 2000). While MARV VP24 has been shown to interfere with the inflammatory responses of its host (Edwards *et al*., 2014; Page *et al*., 2014), no direct role as an IFN antagonist has been reported for this protein to date. A study has shown that the VP24 of EBOV plays an important role in adding genomic RNA to virus particles (Watt *et al*., 2014); however, at the time of writing it is unclear whether the protein serves similar functions in MARV.

1.3.1.7 RNA-dependent RNA polymerase

The L gene of MARV varies by 12% at the amino acid level between different MARV strains (Towner *et al*., 2006) and codes for the L structural protein (267 kDa). This protein performs the enzymatic functions of the virus and functions as an RNA-dependent RNA polymerase, mRNA (guanine-N(7)-)-methyltransferase, poly(A) synthetase and mRNA guanylyl transferase. The L protein is essential for transcription and replication, and may have additional

functions such as RNA synthesis, capping and polyadenylation (reviewed in Brauburger *et al*., 2011).

1.4 Epidemiology of Marburg virus

1.4.1 Case fatality rate

For more than two decades following the initial discovery of MARV, only sporadic outbreaks of MVD occurred, affecting just a small number of people (Table 1.1). The outbreaks were also associated with relatively lower case fatality rates than those seen in outbreaks of EBOV disease (~30% for MARV compared to ~90% for EBOV), and MARV was therefore considered less perturbing than its infamous relative (reviewed in Brauburger *et al*., 2012). This view changed when MARV suddenly re-emerged to cause large outbreaks affecting hundreds of people in the DRC from 1998 to 2000 (Bausch *et al*., 2006), and in Angola from 2004 to 2005 (Towner *et al*., 2006), with case fatality rates of up to 90% (Towner *et al*., 2006). The difference in case fatality rates between these and previous MARV outbreaks has been attributed to the varying availability and quality of medical care between different outbreak locations (Bausch *et al*., 2006), the route of infection (Bausch *et al*., 2006), and possible differences in pathogenicity between different MARV strains (Bausch *et al*., 2006; Geisbert *et al*., 2007; Alves *et al*., 2010; Cross *et al*., 2015; Fernando *et al*., 2015).

1.4.2 Outbreaks and demographic characteristics of Marburg virus disease

At the time of writing, the most recent outbreak of MVD occurred in Uganda in October 2017 and involved three fatal cases (World Health Organisation, 2017). Other recorded outbreaks of MVD in humans are summarised in Table 1.1.

Table 1.1: Known outbreaks of Marburg virus disease in humans

Year	Location	Source of exposure	Number of cases	Number of deaths	Reference
1967	Germany and Serbia via Uganda	Non-human primates	31	7	Siegert, 1972
1975	South Africa via Zimbabwe	Unknown/Possible exposure to bats	3	1	Gear *et al*., 1975; Conrad *et al*., 1978
1980	Kenya	Unknown	2	1	Smith *et al*., 1982
1987	Kenya	Unknown/ Visit to Kitum Cave	1	1	Johnson *et al*., 1996
1988	Russia	Laboratory infection	1	1	Kuhn, 2008
1990	Russia	Laboratory infection	1	0	Nikiforov *et al*., 1994
1998 – 2000	DRC	Contact with bats in mines	154	127	Bausch *et al*., 2006
2004 – 2005	Angola	Unknown	252	227	Towner *et al*., 2006
2007	Uganda	Contact with bats in mines	4	2	WHO, 2007
2008	The USA via Uganda	Visit to Python Cave	1	0	CDC, 2009
2008	The Netherlands via Uganda	Visit to Python Cave	1	1	Timen *et al*., 2009
2012	Uganda	Unknown	18	9	WHO, 2012
2014	Uganda	Unknown	1	1	WHO, 2014
2017	Uganda	Possible entry into a cave, preparation of a body for burial	3	3	WHO, 2017

During the outbreak of MVD in Durba and Watsa, DRC, between 1998 and 2000, 52% of the infected patients were male mine workers (Bausch *et al*., 2006), while in the MVD outbreak in Uige, Angola, 2005, 75.6% of patients with confirmed infection were female (Roddy *et al*., 2010). In an African setting, females are traditionally associated with nursing and caregiving activities (Roddy *et al*., 2010). The Uige outbreak involved high rates of nosocomial transmission and transmission to caregivers, which may explain the higher infection rate amongst females during this outbreak (Roddy *et al*., 2010). In general, no specific trends have been observed regarding the age or sex of infected patients across several different outbreaks of MVD (Bausch *et al*., 2006).

1.5 Marburg virus host range and geographic distribution

Marburg virus disease outbreaks usually occur in the drier savannah areas of central and East Africa, but have also occurred in the more tropical ranges of Southern Africa (Gear *et al*., 1975; Towner *et al*., 2006). The virus has a zoonotic origin, and the initiation of several MVD outbreaks has been associated with entry into caves and mines inhabited by bats (see section 1.4.2, Table 1.1).

1.5.1 Reservoir hosts

Reservoir hosts are living entities that typically maintain a pathogen without noticeable illness, but can transmit these infectious agents to susceptible hosts with often severe health consequences (Schountz, 2014). Several studies have indicated that bats may be possible reservoir hosts for MARV (Swanepoel *et al*., 2007; Towner *et al*., 2007; Towner *et al*., 2009). The ability of bats to migrate via flight, as well as their preference for roosting in large groups, make them ideal candidates for the attainment and maintenance of viruses such as MARV (Allen *et al*., 2008). The first evidence for MARV infection in bats was published in 2007, after MARV RNA was detected in 12 bats collected in the Goroumbwa mine in the DRC (Swanepoel *et al*., 2007), and four bats collected near caves in Gabon (Towner *et al*., 2007). Soon after, MARV was isolated from healthy ERBs (*Rousettus aegyptiacus*) caught in the Kitaka cave in Uganda (Towner *et al*., 2009). The nucleotide sequences of the nucleic acid and isolates collected from these bats were matched closely to those of isolates obtained from humans who were infected with MARV within the same area and in the same year (Swanepoel *et al*., 2007; Towner *et al*., 2009). The ERB has since been confirmed as a reservoir host for MARV based on experimental inoculation studies where bats became infected with and shed the virus, but did not show clinical signs of disease (Paweska *et al*., 2012; Amman *et al*., 2015; Paweska *et al*., 2015; Schuh *et al*., 2017a). It remains to be confirmed whether other bat species may also serve as reservoir hosts for MARV.

Egyptian rousette bats are widespread and are found in the Middle East, the coast of Pakistan, East Africa, central Africa, West Africa, Southern Africa and southwest Asia (Barclay &

Jacobs, 2011). Serological and molecular evidence for natural exposure to or infection with MARV have been reported in ERBs in South Africa (Paweska *et al*., 2018), Uganda (Towner *et al*., 2009; Amman *et al*., 2012), Kenya (Kuzmin *et al*., 2010), the DRC (Swanepoel *et al*., 2007), the Republic of the Congo (Towner *et al*., 2007; Pourrut *et al*., 2009) and Gabon (Towner *et al*., 2007; Pourrut *et al*., 2009). As shown in Figure 1.2, MVD outbreaks in human populations tend to coincide with areas where ERBs occur, and where serological and molecular evidence for the virus have been found in these animals. A study modelling the zoonotic transmission potential of MARV showed that up to 27 African countries, inhabited by more than 100 million people, might be at risk of MVD outbreaks (Pigott *et al*., 2015). The model was based on environmental suitability for zoonotic MARV transmission but unfortunately did not include ERB distribution data due to a lack of differentiation between bat roosting and foraging sites (Pigott *et al*., 2015).

Egyptian rousette bat approximate distribution range
Serological evidence for Marburg virus in Egyptian rousette bats
Molecular evidence for Marburg virus in Egyptian rousette bats
Isolation of Marburg virus from naturally infected Egyptian rousette bats
Marburg virus outbreak/human cases

Figure 1.2: Overlapping geographic distribution of the Egyptian rousette bat and human outbreaks of Marburg virus disease.

The Egyptian rousette bat distribution range is shown as purple shaded areas; areas where serological and molecular evidence for Marburg virus in Egyptian rousette bats were obtained are depicted as red and yellow triangles, respectively; the area where Marburg virus was isolated from naturally infected Egyptian rousette bats is indicated with a blue star; and areas where Marburg virus disease outbreaks have occurred in human populations are indicated with green circles. Egyptian rousette bat distribution data were obtained from the African Chiroptera Report (2017).

1.5.2 Susceptible hosts

Limited information is available on the susceptible host range of MARV. Marburg virus and other filoviruses cause severe disease and often death in humans (*Homo sapiens*) and NHPs (Bermejo *et al*., 2006). Experimental susceptibility to MARV infection has been demonstrated in common marmosets (*Callithrix jacchus*) (Carrion *et al*., 2011), cynomolgus macaques (*Macaca fascicularis*) (Geisbert *et al*., 2007), African green monkeys (*Chlorocebus aethiops*) (Gonchar *et al*., 1991), rhesus macaques (*Macacamulatta*) (Geisbert *et al*., 2002), baboons (*Papio hamadryas*) (Ryabchikova *et al*., 1999) and squirrel monkeys (*Saimiri sciureus*) (Lub *et al*., 1995). Serological studies and experimental infections have suggested the susceptibility of dogs (*Canis lupus familiaris*), duiker antelope (*Sylvicapra grimmia*), ferrets (*Mustela putorius furo*) and pigs (*Sus scrofa domesticus*) to EBOV infection (Rouquet *et al*., 2005; Kobinger *et al*., 2011; reviewed in Weingartl *et al*., 2013; Kozak *et al*., 2016), but it is not yet known whether these animals could also be susceptible to MARV infection. Wild-type MARV infections do not result in fatalities in immunocompetent rodents including mice, hamsters and guinea pigs, but serially passaged virus may produce strains that are lethal to these animals (Warfield *et al*., 2009).

1.6 Transmission of Marburg virus

Marburg virus disease outbreaks are rare, and transmission is therefore most likely an uncommon event, with only restricted contact occurring between susceptible humans and infected reservoir hosts. It has been suggested that MARV transmission may occur from bats to humans by direct contact between mucous membranes or open wounds and bat excreta, bites, hunting and consumption of bats, or consumption of or contact with fruit and other objects contaminated with the saliva, blood, urine or faeces of bats (Swanepoel *et al*., 1996; Leroy *et al*., 2005; Amman *et al*., 2015). Human-to-human transmission chains then follow, and generally occur through direct contact with the body fluids of infected patients while caring for them, or via contact with their remains during traditional burial activities (Roddy *et al*., 2010). Marburg virus appears to favour entry through mucous membranes or cuts and scrapes in the skin, and needle-stick injuries and the re-use of contaminated needles have been the source of

laboratory- and hospital-related filovirus infections (Edmond *et al*., 1977; World Health Organisation, 1978b; reviewed in Silver, 2015).

The transmission pathways for MARV in reservoir host populations remain unclear. In four separate studies where ERBs were experimentally infected with MARV, it was shown that viraemia was present for at least 5 days post-infection (but cleared from the blood by 10-16 days post-infection), which may facilitate transmission from infected bats to susceptible hosts by contact with infected blood, for example during the hunting and slaughter of these animals (Paweska *et al*., 2012; Amman *et al*., 2015; Paweska *et al*., 2015; Schuh *et al*., 2017a). Furthermore, MARV could be detected in a number of tissues including the salivary glands, lungs, kidneys, large intestine, bladder and female reproductive tract, which may facilitate horizontal (reproduction or direct contact with bodily fluids) or vertical (from dam to neonate during birth) transmission between bats (Paweska *et al*., 2012; Amman *et al*., 2015; Paweska *et al*., 2015; Schuh *et al*., 2017a). In one study, MARV was isolated from the saliva of experimentally infected bats on several occasions, indicating a potential for transmission of the virus between bats via biting or licking of mucous membranes, and between bats and other animals and humans via bites and half-eaten fruit (Amman *et al*., 2015). Additionally, Schuh and colleagues (2017a) were able to detect MARV in oral swabs from healthy, previously naive bats that were in contact with MARV-infected bats, providing evidence for horizontal transmission of the virus between these animals.

Although not a likely natural route of transmission, MARV may be transmitted via aerosol exposure under special circumstances, as demonstrated in an experiment conducted by Alves and colleagues (2010). In their study, cynomolgus macaques were exposed to either a high or a low dose of an aerosolised strain of MARV Angola, and all of the animals became febrile within a week post-exposure and eventually succumbed to the infection (Alves *et al*., 2010). The potential for aerosol spread of MARV is worrying, as this increases the risk for this virus to be exploited as a bioterrorism agent, and of transmission to humans whilst entering cave roosts or mines inhabited by ERBs.

1.7 Replication and dissemination of Marburg virus

Information on the replication strategies of MARV is mostly limited to studies conducted using recombinant systems such as minigenomes and VLPs, and the results of the majority of these studies are yet to be reproduced with infectious virus. Marburg virus has an extended cell tropism and may infect a variety of cells. Upon infection of a susceptible host, the MARV GP attaches to a suitable host cell by interacting with cell surface proteins and is subsequently endocytosed (Manicassamy *et al*., 2007). The MARV GP is then proteolytically cleaved into two subunits (GP_1 and GP_2) (Misasi *et al*., 2012) and pH-dependent fusion occurs with the assistance of GP_2 (Weissenhorn *et al*., 1998). The MARV nucleocapsid is subsequently released into the host cell cytoplasm, inclusion bodies are formed, and transcription and translation of the MARV genome takes place (reviewed in Brauburger *et al*., 2012; reviewed in Kajihara & Takada, 2015). Following assembly of the newly synthesised virus particles, the VP40 recruits NP, GP and VP24 to the cell membrane, and budding of the virus occurs (Mittler *et al*., 2007; Dolnik *et al*., 2010).

Marburg virus replicates in macrophages, monocytes, fibroblasts, hepatocytes, endothelial cells, epithelial cells and dendritic cells, with macrophages, monocytes and dendritic cells being the replication and dissemination sites of choice (Skripchenko *et al*., 1994; Geisbert *et al*., 2003; Fritz *et al*., 2008; Hensley *et al*., 2011). From these cells, the virus spreads to the local lymph nodes, the liver, the spleen and the adrenal glands (Geisbert *et al*., 2003; Hensley *et al*., 2011). The adrenal glands play an essential role in regulating blood pressure, and infection of these glands lead to the hypotension, hypovolaemia and shock that are frequently reported during the late stages of MARV disease (Feldmann *et al*., 2013).

1.8 Marburg virus immune evasion strategies and pathogenesis

To ensure proliferation, viruses must usually infect their hosts without causing significant disease and be transmitted before being eliminated by the host's immune system (Schountz, 2014). Several viruses have therefore evolved immune evasion strategies that alter the immune responses of their hosts to favour their own survival (Schountz, 2014). The immune evasion strategies of filoviruses rely on accessory proteins that are evolutionarily adapted to their

reservoir hosts (Schountz, 2014). These proteins often behave differently in incidental hosts and may promote viral pathogenesis (Schountz, 2014). While the immune evasion strategies of EBOV have been studied extensively, much less is known for MARV. During the early stages of infection, EBOV inhibits the host innate immune response by disguising epitopes, down-regulating type I IFN responses and undermining the humoral immune response by secreting a non-structural protein, sGP, unique to EBOV, which acts as a decoy (reviewed in Wong *et al*., 2014). Ebola virus GP is known to have antagonistic activity to tetherin, a cell surface protein that blocks the release of enveloped viruses from the infected host cell (Kaletsky *et al*., 2009; Kühl *et al*., 2011). Additionally, densely clustered glycans on the EBOV GP may shield the viral surface from antibody surveillance (Cook & Lee, 2013). It is unclear whether MARV GP has similar abilities. The MARV VP40 inhibits the phosphorylation of Janus kinase (JAK) and signal transducer and activator of transcription (STAT), which impairs host immunity during infection by inhibiting the transcription of IFN-stimulated genes (Valmas *et al*., 2010). The VP35 has been shown to function as an IFN antagonist, and expression of VP35 alone has been shown to be sufficient to block the production of IFN-α in human dendritic cells (Bosio *et al*., 2003). Studies conducted on the functions of EBOV VP35 have shown that this protein may inhibit RNA silencing pathways, as well as prevent the activation of an antiviral protein, protein kinase R (Hartman *et al*., 2004). Marburg virus VP35 could serve similar functions, as the IFN inhibitory domain of the VP35 protein of MARV is identical to that of EBOV (reviewed in Brauburger *et al*., 2012). Furthermore, MARV VP35 has the ability to cap the ends of double-stranded RNA, hiding it from the immune system and preventing the production of IFN-α and β (Bale *et al*., 2012a; reviewed in Audet & Kobinger, 2015).

1.9 Immunity and host immune responses to Marburg virus infection

The mammalian immune system consists of both innate (non-specific) and adaptive (specific) elements. Innate immunity provides the first line of defence against invading pathogens such as viruses (Allen *et al*., 2008). Central aspects of the innate immune system include the IFN response, complement, inflammation (mediated by cytokines including tumour necrosis factor (TNF) and interleukin 1), and innate immune cells (leukocytes and phagocytes). Innate immune cells express pathogen recognition receptors (PRR) (Toll-like receptors (TLRs), RIG-like receptors (RLRs) and nonobese diabetic-like receptors (NLRs)) which identify pathogen-

associated molecular patterns (PAMPs) that are evolutionarily conserved in viruses and other pathogens (Baker & Zhou, 2015), and initiate antigen-specific adaptive immune responses (Kumar *et al*., 2011). The IFN response confers an antiviral state to cells in order to prevent the spread of infection (Randall & Goodbourn, 2008). To date, three classes of IFN have been discovered (type I, II and III), of which two (types I and III) are directly activated by viruses to induce antiviral activity through IFN stimulated genes (Sadler & Williams, 2008). Type II IFNs play a role in activating macrophages and inducing the expression of class I major histocompatibility complex molecules (Kuzmin *et al*., 2017).

Adaptive immune responses are controlled by T and B lymphocytes, and are integral in the clearing of infections and in long-lasting protection against pathogens (Allen *et al*., 2008). Immunoglobulins (or antibodies) are secreted by B lymphocytes and contain variable, diversity and joining gene segments that may recombine to produce a large variety of specific antibodies (termed combinatorial diversity) (Schountz *et al*., 2017). Antibodies serve several important functions in the adaptive immune system, including the neutralisation, precipitation, agglutination and opsonisation of antigens, and the triggering of cytotoxicity and complement pathways (reviewed in Baker *et al*., 2013). Antibody responses are categorised as being either active or passive. Passive immunity is usually short-lived and consists of antibodies obtained from an immune donor (acquired), or transferred from a mother to an infant across the placenta (natural) (Abbas *et al*., 2014). Active immunity requires exposure to an antigen, and can be obtained via natural infection, or acquired through vaccination (Abbas *et al*., 2014). Active immunity typically provides long-term and sometimes life-long protection against a specific pathogen. However, in some cases, immunity may wane over time, leading to susceptibility to reinfection (Wendelboe *et al*., 2005). Cell-mediated adaptive immunity is governed by cluster of differentiation 8 (CD8) cytotoxic and cluster of differentiation 4 (CD4) helper T lymphocytes. These cells assist in destroying virus-infected cells and activate antibody and cytokine responses (Baker & Zhou, 2015).

1.9.1 Immune responses of incidental hosts

Information on the immune responses of incidental hosts to filovirus infection is mostly limited to studies conducted in humans infected with EBOV or SUDV, or experimental studies conducted in rodents and NHPs. Fatal filovirus infections usually involve the unregulated release of chemokines and inflammatory mediators from infected cells along with broad T and B lymphocyte apoptosis (Leroy *et al*., 2000; Leroy *et al*., 2001; Baize *et al*., 2002; Connor *et al*., 2015). In animal models, regulated cellular and humoral immune responses are essential for protection against lethal filovirus disease (Wilson *et al.*, 2000; Bray *et al*., 2001; Parren *et al*., 2002; Takada *et al*., 2007a).

1.9.1.1 Innate immune responses of incidental hosts

The innate immune responses of humans and other animals against MARV are poorly studied. Studies in NHPs have indicated that robust, but delayed innate immune responses develop upon experimental infection with MARV (Fritz *et al*., 2008; Connor *et al*., 2015; Fernando *et al*., 2015). The cytokine storm typical of MARV infection was present in all infected animals in these studies, with IFN-γ, TNF, C-C motif chemokine ligand 2 (CCL2) and interleukin 6 levels increasing in the blood (Fernando *et al*., 2015), and natural killer (NK) cell levels rapidly depleting within the first week after infection (Fritz *et al*., 2008; Fernando *et al*., 2015). The type I IFN response seems to be essential for controlling MARV infection in mice (Bray, 2001). However, infection with MARV produces a robust type I IFN response in the liver and spleens of both lethal hamster models (Marzi *et al*., 2016) and NHPs that succumb to the disease (Connor *et al*., 2015), suggesting that IFN responses are not necessarily protective against MARV in these animals.

1.9.1.2 Adaptive immune responses of incidental hosts

In survivors of filoviral disease, high titers of antibodies are produced during the early stages of infection (approximately 14 days after the onset of symptoms), while antibody titers are mostly low or non-existent in those who do not survive (Baize *et al*., 1999; Sobarzo *et al*., 2012; Sobarzo *et al*., 2013). Therefore, the antibody response may be vital in protecting humans

against MVD. A study by MacNeil and colleagues (2011a) using specimens from patients who survived infection with different ebolavirus species, showed that immunoglobulin M (IgM) antibody titers were detectable from 2 days after the onset of symptoms, peaked between 30 and 50 days after the onset of symptoms, and started to decline after approximately 80 days. Immunoglobulin G (IgG) antibodies were present from the early stages of infection (day 6 to day 18 after the onset of symptoms), and titers remained high throughout the course of specimen collection (approximately four months after the onset of symptoms) (MacNeil *et al*., 2011a). Later studies showed that IgG antibodies to SUDV, MARV and EBOV may persist in human survivors for 11 (Sobarzo *et al*., 2013; Natesan *et al*., 2016) to 40 years (Rimoin *et al.*, 2018) following infection.

A study conducted by Taniguchi and colleagues (2012) on the antibody responses of cynomolgus macaques naturally infected with RESTV, indicated that antibodies directed against the GP were concurrent with neutralisation activity and viral clearance. Epitopes in the GP_1 and GP_2 of EBOV have been shown to be conformation-dependent, and antibodies identifying these epitopes neutralise the virus by inhibiting cathepsin cleavage of the GP or recognising the GP in cleaved form (Shedlock *et al.*, 2010). Marburg virus neutralising antibodies apparently inhibit the virus by binding to an exposed Niemann-Pick C1 receptor-binding site on the GP (Flyak *et al*., 2015).

Neutralising antibody responses to MARV in humans are rare, weak and short-lived. Stonier and colleagues (2017) showed that only two out of six survivors of MVD tested in their study produced neutralising antibodies to the virus. Neutralising antibody titers did not exceed 1:40 and diminished by 27 months post-infection despite sustaining high overall antibody titers (Stonier *et al*., 2017). Similarly, neutralising antibodies produced by cynomolgus macaques vaccinated against MARV also decreased over time (Mire *et al*., 2014). Furthermore, Sobarzo and colleagues (2016) showed that survivors of SUDV disease did not produce persistent neutralising antibody responses, and several survivors lacked memory humoral immunity completely. Where neutralising immune responses were present, high levels of cytokine and chemokine responses were consistently recorded (Sobarzo *et al*., 2016). The results from these studies suggest that filovirus infection might not induce life-long sterilising immunity in humans and NHPs. However, it is possible that mechanisms other than neutralisation can be

involved in protection against reinfection, and as of 2018, there is no evidence to suggest that previously exposed humans and NHPs are protected against reinfection with filoviruses, or to suggest the contrary.

Cell-mediated immune responses against MARV seem to rely on memory CD4 T lymphocytes rather than CD8 T lymphocytes, and Stonier and colleagues (2017) showed that anti-MARV CD4 T cell responses are present in survivors for at least 2 years post-infection. These findings are similar to results obtained by Sobarzo and colleagues (2016) from testing humans infected with SUDV. However, in both studies, specimens were collected from survivors several months after recovering from the respective diseases. Sanchez and colleagues (2004) previously detected active CD8 T cell responses in survivors of SUDV shortly after exposure to the virus, and these responses have also been shown to be present in both survivors and victims of EBOV disease (McElroy *et al*., 2015; Agrati *et al*., 2016; Ruibal *et al*., 2016). It is, therefore, possible that CD8 T cell responses could have been present shortly following MARV and SUDV infection, but had diminished by the time specimens were collected in the respective studies. The testing of acute specimens in future outbreaks will shed more light on the role of cell-mediated immunity in humans shortly after MARV infection.

1.9.2 Immune responses of reservoir hosts

Bats are natural reservoirs for several viruses, including filo-, corona-, lyssa- and paramyxoviruses (Luis *et al*., 2013). Bats live long lives (10 to 30 years) compared with mice and other mammals of similar sizes (one to three years), and due to exceptional immunity, may remain relatively disease-free for the majority of their lives (Zhang *et al*., 2013). More than 100 virus species have been detected in bats, of which only a few (Tacaribe virus, rabies virus, the Australian bat lyssavirus and other rabies-related lyssaviruses) have been shown to cause disease in these animals thus far (reviewed in Baker *et al*., 2013). O'Shea and colleagues (2014) suggested that the absence of disease in bats could be explained by the elevated body temperature of these animals during flight, which could mimic the effect of fever and result in limited viral replication. However, another study showed that bat cells were able to support replication of EBOV despite higher incubation temperatures (Miller *et al*., 2016), indicating that elevated body temperature alone is unlikely to be sufficient to control viral replication.

More likely, the ability of bats to host viruses without displaying signs of disease is a result of a delicate balance between the bat innate and adaptive immune systems and viral infection.

1.9.2.1 Innate immune responses of reservoir hosts

The innate immune responses of bats to filovirus infection are largely unknown as there are no reagents or assays available yet to monitor these responses in these animals. However, full genome annotations of 11 bat species (*Rousettus aegyptiacus* (Pavlovich *et al*., 2018), *Myotis rufoniger* (Bhak *et al*., 2017), *Rhinolophus ferrumequinum*, *Megaderma lyra*, *Pteronotus parnellii*, *Eidolon helvum* (Parker *et al*., 2013), *Myotis brandtii* (Seim *et al*., 2013), *Pteropus alecto*, *Myotis davidii* (Zhang *et al*., 2013), *Pteropus vampyrus* and *Myotis lucifugus* (Lindblad-Toh *et al*., 2011)) and the transcriptomes of several bat species including *Rousettus aegyptiacus* (Lee *et al*., 2015), *Pteropus alecto* (Papenfuss *et al*., 2012) and *Arbiteus jamaicensis* (Shaw *et al*., 2012)) have become available and have shed light on the genes involved in bat innate immunity. Analyses of these genomes and transcriptomes suggest that bats share several aspects of their innate immune systems with humans and other mammals (Baker & Zhou, 2015; Schountz *et al*., 2017). Type I, II and III IFNs have been identified in a few bat species including *Rousettus aegyptiacus* and *Pteropus vampyrus* (He *et al*., 2010; Kepler *et al*., 2010; Pavlovich *et al*., 2018). Additionally, TLRs and cytokines highly similar to those of other mammals have been identified in two bat species (*Pteropus alecto* and *Rousettus leschenaultii*) (Iha *et al*., 2009; Iha *et al*., 2010; Cowled *et al*., 2011), and RLRs and NLRs have been reported in *Pteropus alecto* (Papenfuss *et al*., 2012). The STAT1 signalling pathway in ERBs has been shown to be comparable to that of other mammals (Fujii *et al*., 2010). The STAT1 is a transcription factor involved in upregulating immunity-related genes in response to IFN stimulation (Dupuis *et al*., 2003).

Even though several similarities exist between the immune systems of bats and other mammals, many differences have been observed that may be related to the non-pathogenic characteristics of most viral infections in bats (reviewed in Baker *et al*., 2013). For example, type II and III IFN receptors have a broader tissue distribution in bats compared to humans, suggesting a more prominent role for these IFNs in bat immunity (Zhou *et al*., 2011a; Zhou *et al*., 2011b). In addition, aspects of the type I IFN system have been shown to be constitutively active in

Pteropus alecto (Zhou *et al*., 2016). This constitutive IFN activity may allow bat cells to respond instantly to viral infection, which may limit, but not entirely inhibit, viral replication (Schountz *et al*., 2017). In a study comparing the innate immune responses of human and ERB cells to filoviruses, Kuzmin and colleagues (2017) showed that MARV infection initiated a stronger innate immune response in the bat cells compared to human cells. Moreover, IFN-α, -β (type I IFNs) and –γ (type II IFN) controlled filovirus infection in bat cells, but only type I IFNs controlled filovirus infection in human cells (Kuzmin *et al*., 2017). Several studies have reported a noticeable absence or decrease in receptors for NK cells in bats (Shaw *et al*., 2012; Zhang *et al*., 2013; Lee *et al*., 2015); however, a more recent study has identified a diversified and expanded family of NK receptors in the ERB genome (Pavlovich *et al*., 2018). The difference in observations could possibly be ascribed to the low-coverage sequencing techniques utilised by the former studies. Hölzer and colleagues (2016) reported that MARV replicates more rapidly in human hepatoma cells compared to ERB embryonic cells based on viral RNA levels shortly following infection. This observation suggests that bats have more time to activate and establish innate immune defence mechanisms upon MARV infection.

Zhang and colleagues (2013) hypothesised that the evolution of the deoxyribonucleic acid (DNA) repair response for flight might have enhanced bat innate immune responses. Bats and viruses have co-existed for millions of years, which most likely guided evolutionary changes in the genes related to the bat innate immune response and the mechanisms that control viral replication (Zhang *et al*., 2013). In bats, the genes involved in the repair of DNA damage evolved the fastest, which enabled bats to fly (Zhang *et al*., 2013). The DNA repair response also plays several roles in the innate immune system, and is a frequent target for virus activities (Zhang *et al*., 2013). Infection of cells with RNA viruses may result in the release of host DNA into the cytoplasm, which activates the DNA repair response in mammals and triggers robust type I IFN responses by the activation of stimulator of IFN genes (STING) (Ryan *et al*., 2016; Schlee & Hartmann, 2016). The extreme physical effort and high metabolic activity required for sustained flight in bats generates reactive oxygen species, which results in similar tissue damage and cytosolic DNA (Shen *et al*., 2010; Zhang *et al*., 2013). Xie and colleagues (2018) showed that a highly conserved serine residue (S358) in the STING of mammals is replaced in bats, which results in a dampened IFN response in these animals. Furthermore, Ahn and colleagues (2016) showed that the PYHIN gene family, involved in inflammasome activation

and DNA sensing, is lost in all genomes available for bats at the time of writing. These studies indicate that bats evolved specialised mechanisms to prevent overactivation of the innate immune system during flight, which, in turn, allowed viruses to coexist within these animals. Innate immunity might therefore be important for controlling MARV infection in bats; however, the exact mechanisms involved remain to be determined through experimental infection studies.

1.9.2.2 Adaptive immune responses of reservoir hosts

Bats share several aspects of their humoral immune system with other mammals. B cells and T cells similar to those of humans and mice have been observed in the spleens and lymph nodes of Indian fruit bats (*Pteropus giganteus*) and in histological sections from Brazilian free-tailed bats (*Tadarida brasiliensis*) (Chakravarty & Sarkar, 1994; Turmelle *et al*., 2010). In addition, some species of bats have been shown to produce IgM, immunoglobulin A (IgA) and IgG that are homologous to human immunoglobulins (Chakravarty & Sarkar, 1994; Butler *et al*., 2011). However, bats seem to possess more variable, diversity and joining gene segments than humans do, and may therefore be capable of generating a much more extensive collection of naive immunoglobulins by combinatorial diversity (Baker & Zhou, 2015; Schountz *et al*., 2017). This may allow bats to respond rapidly to viral infection without the need for antibody affinity maturation (Schountz *et al*., 2017).

Plowright and colleagues (2016) hypothesised that bats could obtain life-long humoral immunity after primary infection with a virus, or lose immunity and become susceptible to reinfection. It is also possible that filoviruses may persist as a latent infection in the reservoir host, and then become activated through stimuli such as stress, pregnancy, co-infections or a combination of these factors (Gupta *et al*., 2004; Plowright *et al*., 2016). Immune suppression has been shown to be associated with stress in a few mammalian species (reviewed in Nelson *et al*., 2000); however, evidence linking stress with immune function in bats is limited. During pregnancy, changes in the immune responses of bats seem to favour viral replication, with increased viral titers in the blood, placenta and birthing fluids bearing an increased risk of infection to humans and other animals (Leroy *et al*., 2005). Roost type and the environment (Allen *et al*., 2008; Epstein *et al*., 2013) may also influence antibody responses in bats.

The maintenance of viruses in bat colonies requires a contant influx of susceptible hosts through the loss of active or passive immunity, or migration of bats between colonies (Plowright *et al*., 2016). A study conducted in fruit bats on the longevity of maternal antibodies against canine distemper virus and Hendra virus has shown that antibodies are transferred from dams to pups, and that these antibodies may be present in pups for approximately 8 months after birth (Epstein *et al*., 2013). The duration of maternal immunity to MARV in ERBs is currently unknown, although it has been suggested that maternal antibodies might persist between 3 and 5 months in juvenile bats (Paweska *et al*., 2015). Paweska and colleagues (2012) performed an experimental infection study of ERBs with MARV by various inoculation routes. The study showed that IgG antibodies against MARV were present at days 9 to at least 21 post-infection (Paweska *et al*., 2012), and these results were confirmed in later studies by the same and other groups (Amman *et al*., 2015; Paweska *et al*., 2015). Schuh and colleagues (2017a) reported a rapid decline in IgG levels in MARV-infected ERBs within 1 month after seroconversion, with the antibodies becoming undetectable by the third month post-infection. Results of a single study have indicated that ERBs develop low levels of neutralising antibody against MARV (Paweska *et al*., 2012), but it remains unclear whether antibodies are protective against reinfection, viral replication and shedding (Paweska *et al*., 2012; Schuh *et al*., 2017a). The seemingly poor neutralising antibody responses of bats might contribute to persistent infection and shedding in these animals and might lead to viral reactivation when antibody levels decrease (Schountz *et al*., 2017).

Cell-mediated adaptive immune responses are poorly studied in bats (Baker & Zhou, 2015). Transcriptome analyses have indicated that receptors that are present on the T lymphocytes of other mammals are conserved in bats (Papenfuss *et al*., 2012; Shaw *et al*., 2012), and the co-receptor for CD4 T lymphocytes has been identified in ERBs (Omatsu *et al*., 2008). The exact role of the T cell responses of bats in viral infection remains to be determined.

Despite the latest advances in knowledge, bat immunity remains poorly understood, and little is known about the immune responses elicited upon infection of ERBs with MARV. Understanding the immune responses of bats, particularly those living in close proximity to humans, is crucial in identifying the viral dynamics in potential reservoir bat populations, and

in turn, the risk of spread to humans and other animals (Epstein *et al*., 2013). For this reason, there is a need for the development of methods to characterise and evaluate the immune responses of bats.

1.10 Identification of Marburg virus

Due to the high lethality of MARV, the unavailability of a suitable licensed vaccine and the potential for aerosol spread, the virus is classified as a Category A biological threat by the National Institute of Allergy and Infectious Diseases, a Risk Group 4 agent by the World Health Organisation (WHO) and a Select Agent by the Centres for Disease Control and Prevention (CDC) (reviewed in Brauburger *et al*., 2012). The highest level of containment (biosafety level 4 (BSL-4)), as well as specialised packaging and shipping procedures for specimens are therefore required in order to diagnose and research MVD. The diagnosis of MVD in humans largely relies on clinical symptoms and patient history (geographic location, travel, entry into caves or mines and exposure to bats or other infected animals such as NHPs), but specific diagnostic tests are essential in providing a definitive diagnosis. At the time of writing, diagnostic methods available for MARV and other filoviruses include virus isolation, antigen detection tests, serological tests and reverse transcription polymerase chain reaction (RT-PCR) assays in either conventional or real-time format. Marburg virus infections are diagnosed and investigated by the Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD) of the National Institute for Communicable Diseases of the National Health Laboratory Service (NICD-NHLS), South Africa, which is a WHO Collaborating Centre for Research and Reference on Viral Haemorrhagic Fevers (VHF) and Arboviruses in Africa. Although rare, MVD cases may arise in South Africa from travellers passing through or returning to the country from endemic African countries. With increased and rapid travel within and between continents, the potential importation of MARV and other filoviruses to non-endemic countries is concerning. Initial filovirus disease symptoms may resemble the symptoms of other more prominent diseases such as malaria, typhoid fever and yellow fever (Isaacson *et al*., 1978; Feldmann & Klenk, 1996; Siegenthaler, 2007). Additionally, filoviruses are known to bear a high risk of nosocomial transmission with a high fatality rate amongst healthcare workers, weakening public health systems (Vanessa & Matthias, 2012). As of 2018, there are no WHO-approved diagnostic tests for MVD. The NICD-NHLS and other WHO VHF reference

laboratories currently rely on in-house real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) methods targeting either the L (Panning *et al*., 2007) or VP40 gene of MARV, and in-house serological assays based on the methods described by Ksiazek and colleagues (1999) (Knust *et al*., 2015) for the diagnosis of MARV in humans. These methods are also used for surveillance for MARV in bat populations in Africa.

With environmental and climatic changes, an increasing population and an escalating demand for land, urbanisation and international trade and travel, the risk of coming into contact with MARV and its reservoir hosts is becoming significantly higher (Karesh *et al*., 2012). For this reason, it is crucial that improved surveillance tools are developed and efforts implemented for monitoring MARV in wild bat populations before they spill over into humans and other animals. With improved surveillance, the initiation of an outbreak can be identified at an early stage and outbreak response measures can rapidly be applied in the affected locations, restricting the spread and impact of MVD.

1.10.1 Serological assays for the detection of antibodies to Marburg virus

To date, the use of serology in MARV diagnostics and surveillance in both humans and animals has been problematic. Traditional enzyme-linked immunosorbent assay (ELISA) methods are based on crude reagents such as antigens that have been prepared in the form of infected cell lysate or cell slurry (Ksiazek *et al*., 1999). The use of crude antigens introduces the risk of detecting non-MARV specific antibodies, resulting in reduced specificity and false-positive results. In addition, no serological assays for MARV have been validated for use with human or animal sera, predominantly due to the unavailability of large enough panels of sera from infected individuals. The validation and standardisation of assays is crucial to compare the efficacy of these tests against different filovirus species, as well as to compare results between different laboratories (Shurtleff *et al*., 2012).

Despite the aforementioned problems with serological assays, serology is of the utmost importance in the diagnosis of MVD in Africa. Outbreaks of MVD generally occur in far-off, inaccessible and politically unstable locations where limited resources exist, and specimens are often only collected following viral clearance or stored in unfavourable conditions, reducing

the possibility of using molecular diagnostic assays such as qRT-PCR (MacNeil *et al*., 2011b). Rapid, but more importantly accurate diagnosis of MARV infection in a suspected case is of paramount importance to the public health response during a suspected or ongoing outbreak. Furthermore, serological assays are imperative in surveillance efforts in both animal and human populations as they may assist in providing evidence of exposure to MARV, monitoring the immune status of a population against MVD, identifying susceptible target populations, and predicting and managing possible spillover events and future outbreaks. The serological assays available for the detection of antibodies to MARV are summarised in Table 1.2. A further discussion on each type of assay is provided in the sections that follow.

Table 1.2: Serological assays for the detection of anti-Marburg virus antibodies

Test	Target antigen	Control antigen	Assay cut-off	Reported use	Limitations	Reference
IF	Lysate of Vero E6 cells inoculated with MARV, EBOV and Lassa virus	None	Not reported	Detection of antibodies to MARV, EBOV and Lassa virus in infected patients	Time-consuming; concerns about specificity; restricted to BSL-4 laboratories; neutralising antibodies to MARV are rare	Johnson *et al*., 1981
PRNT	MARV (Ci-67; Uganda)-inoculated Vero E6 cells	None	Threshold of ≥50% neutralisation of MARV	Detection of neutralising antibodies to MARV	Time-consuming; not validated; restricted to BSL-4 laboratories; neutralising antibodies to MARV are rare	Moe *et al*., 1981; Swenson *et al*., 2008; Flyak *et al*., 2015; Stonier *et al*., 2017
I-ELISA	Lysate of Vero E6 cells inoculated with MARV (various strains)	Lysate of mock-inoculated Vero E6 cells	Threshold differs for each study: threshold for seropositivity was set at mean OD or PP plus 3 SD of negative sera used in each study	Detection of antibodies to MARV in humans infected with the virus; MVD diagnosis; detection of anti-MARV antibodies in bats	Presence of non-specific proteins in lysate may lead to reduced specificity, assay background and false positive results	Ksiazek *et al*., 1999; Bausch *et al*., 2006; Swanepoel *et al*., 2007; Towner *et al*., 2007; Pourrut *et al.,* 2009; Paweska *et al*., 2012; Flyak *et al*., 2015

Test	Target antigen	Control antigen	Assay cut-off	Reported use	Limitations	Reference
I-ELISA	Recombinant MARV NP expressed in *E. coli* and baculovirus expression systems	None	Threshold for seropositivity set at mean OD plus 3 SD of 48 negative control sera	Detection of antibodies to MARV in sera collected from MARV-infected humans and animals	Proteins expressed in *E. coli* are often misfolded and are biologically inactive; very low level of expression of MARV NP in baculovirus system; not validated	Saijo *et al*., 2001a
I-ELISA	Recombinant MARV Angola GP expressed in mammalian cells	Supernatant from cells transfected with plasmid without MARV GP insert	Threshold for seropositivity set at mean OD plus 3 SD of negative control sera	Evaluation of immune responses of mice immunised with MARV VLP and humans infected with MARV	Not validated	Nakayama *et al*., 2010
I-ELISA	Commercially available recombinant MARV Musoke GP (Integrated Biotherapeutics) expressed in mammalian cells	Commercially available recombinant EBOV GP (Integrated Biotherapeutics) expressed in mammalian cells	Threshold for seropositivity set at an OD value of 0.72 based on mean corrected sum OD of 210 juvenile ERBs plus 3 SD	Detection of antibodies to MARV in naturally infected bats	High cost associated with commercially available recombinant antigens; not validated	Amman *et al*., 2012
VNT	Live MARV-MHK	None	Threshold set at ≥75% reduction in cytopathic effect	Detection of MARV neutralising antibody in experimentally infected bats	Time-consuming; not validated; restricted to BSL-4 laboratories; neutralising antibodies to MARV are rare	Paweska *et al*., 2012
I-ELISA	Recombinant MARV NP expressed in *E. coli*	None	Threshold for seropositivity set at mean plus 3 SD of OD values obtained from negative human sera	Detection of antibodies to MARV in negative human sera and sera from MARV immunised rabbits	Not validated; proteins expressed in *E. coli* are often misfolded and are biologically inactive	Huang *et al*., 2014

Test	Target antigen	Control antigen	Assay cut-off	Reported use	Limitations	Reference
Microarray	Recombinant MARV Musoke GP expressed in insect and mammalian cells, MARV Musoke GP, NP and VP40 expressed in *E. coli*, whole inactivated MARV Musoke	None	Not reported	Evaluation of immune responses of MARV survivors and NHPs vaccinated with MARV VLPs	No cut-off reported; not validated	Kamata *et al*., 2014; Natesan *et al*., 2016
I-ELISA	Recombinant MARV Angola NP expressed in *E. coli*	Recombinant RESTV NP expressed in *E. coli*	Threshold for seropositivity set at an OD value of 0.95 based on mean corrected sum OD of ERB breeding colony plus 3 SD	Monitoring of anti-MARV antibody levels in experimentally infected bats	Proteins expressed in *E. coli* are often misfolded and are biologically inactive; not validated	Amman *et al*., 2015; Schuh *et al*., 2017a
I-ELISA	Commercially available recombinant MARV Musoke GP (Integrated Biotherapeutics) expressed in mammalian cells	Commercially available recombinant EBOV GP (Integrated Biotherapeutics) expressed in mammalian cells	Percent positivity of 16.78 in relation to the positive control serum based on the mean PP value plus 3 SD in 15 juvenile ERBs born in captivity	Monitoring of anti-MARV antibody levels in naturally or experimentally infected bats	High cost associated with commercially available recombinant antigens; not validated	Paweska *et al*., 2015; Paweska *et al.*, 2018
I-ELISA	Irradiated MARV Ci67, recombinant MARV GP, HEK293T cell lysate expressing MARV NP, VP24 and VP35	None	Not reported	Evaluation of immune responses of MARV survivors	No cut-off supplied; not validated	Stonier *et al*., 2017
Pseudovirion neutralisation assay	Vesicular stomatitis virus-luciferase pseudovirion with MARV Angola GP surface protein	Pseudovirions with Machupo virus envelope proteins	Not reported	Detection of neutralising antibodies to MARV in immunised NHPs	Not validated	Callendret *et al*., 2018

Abbreviations: IF – immunofluorescent assay; MARV – Marburg virus; EBOV – Ebola virus; BSL-4 – biosafety level 4; PRNT – plaque reduction neutralisation test; I-ELISA – indirect enzyme-linked immunosorbent assay; OD – optical density; PP – percentage positivity; SD- standard deviation; MVD – Marburg virus disease; NP – nucleoprotein; GP – glycoprotein; *E.coli* – Escherichia coli; VLP- virus-like particle; MHK – Michael Hogan kidney strain; NHP – non-human primate; VP – viral structural protein; ERB – Egyptian rousette bat; HEK – human embryonic kidney

1.10.1.1 Marburg virus proteins important for serological assays

Amongst the MARV proteins, the GP and NP are most frequently employed as antigens in serological assays for the detection of MARV-specific antibodies (Sanchez *et al*., 1998; Nakayama *et al*., 2010). Only a limited amount of antibodies is known to neutralise filoviruses, and the majority of these target epitopes on the GP, suggesting that this protein is central to virus neutralisation (Maruyama *et al*., 1999; Wilson *et al*., 2000; Takada *et al*., 2007a; Takada *et al*., 2007b; Bale *et al*., 2012b). The GP has been suggested to be more species-specific compared to other filovirus structural proteins due to a greater genetic variation within this gene (Nakayama *et al*., 2010). Glycoproteins are therefore frequently used in serological assays in which the filovirus species with which a patient is infected, needs to be determined. The NPs of ebolaviruses and marburgviruses have been shown to have comparable amino acid sequences at the N-termini of the proteins (Sanchez *et al*., 1992). Enzyme-linked immunosorbent assays using recombinant NP antigens of EBOV have successfully detected NP-specific antibodies in animals infected with EBOV, SUDV, Taï Forest virus (TAFV) and Reston virus (RESTV), demonstrating strong cross-reactivity for this protein between the different ebolavirus species (Saijo *et al*., 2001a). While some cross-reactive antibody responses have been shown to occur between the NPs of ebolaviruses and marburgviruses, the greatest amount of cross-reactivity occurred between the NPs of viruses within the same genus (Natesan *et al*., 2016). For this reason, NP antigens are useful in detecting genus-specific antibodies, and may be employed in filovirus screening tests (Saijo *et al*., 2001a; Nakayama *et al*., 2010).

A study conducted by Sobarzo and colleagues (2012) has shown that individual recombinant proteins may be valuable for use in filovirus serology, and that making use of serological assays utilising at least two different recombinant antigens may assist in diagnosing filoviral disease more accurately. The study indicated that SUDV proteins NP, GP and VP40 elicit the greatest immunoreactive response in survivors of SUDV disease (Sobarzo *et al*., 2012) (Table 1.3). Results of a subsequent study by Sobarzo and colleagues (2013) differed slightly, with SUDV NP, GP and VP30 being the most immunoreactive (Table 1.3). Similar to findings in survivors of SUDV disease, Stonier and colleagues (2017) showed that survivors of MVD generated robust IgG antibody responses against GP and NP. Immunoglobulin G antibodies were also

detected against VP40 and VP30, but not against VP24 or VP35 (Stonier *et al*., 2017) (Table 1.3). A study by Becquart and colleagues (2014) investigating the human B-lymphocyte epitopes on the GP, NP, VP35 and VP40 of EBOV has shown that serum from persons with an asymptomatic EBOV infection was strongly immunoreactive against VP40, whereas serum from symptomatic survivors reacted strongly with GP (Table 1.3). In the same study, it was suggested that VP35 might be one of the main targets for the humoral immune response in EBOV-infected patients. However, an earlier study investigating the immunogenicity of recombinant EBOV VP35 showed that this protein was of little value for use in serological assays (Groen *et al*., 2003) (Table 1.3). A summary of the immunoreactivity of patient sera in each of the studies described above is provided in Table 1.3. The majority of evidence suggest that the NP and GP are the most suitable antigens for use in serological assays for MARV and other filoviruses, while VP35 and VP24 are ineffectual for this purpose.

Table 1.3: Immunoreactivity of patient sera from different studies against specific filovirus proteins

Filovirus	Patient group	Immunoreactivity of patient sera* (number of sera reactive/number of sera tested)						Reference
		GP	NP	VP40	VP35	VP30	VP24	
Ebola virus	Suspected	NT	16/55	NT	3/55	NT	NT	Groen *et al*., 2003
Sudan virus	Survivors	26/54	27/54	8/54	NT	7/54	NT	Sobarzo *et al*., 2012
	Deceased	0/12	3/12	1/12	NT	0/12	NT	
	Total	26/66	30/66	9/66	NT	7/66	NT	
Sudan virus	Survivors	96/174	112/174	42/174	NT	76/174	NT	Sobarzo *et al*., 2013
	Deceased	1/12	3/12	1/12	NT	0/12	NT	
	Total	97/186	115/186	43/186	NT	76/186	NT	
Ebola virus	Symptomatic	15/21	8/21	21/21	8/21	NT	NT	Becquart *et al*., 2014
	Asymptomatic	17/21	11/21	16/21	12/21	NT	NT	
	Total	32/42	19/42	37/42	20/42	NT	NT	
Marburg virus	Survivors	8/8	8/8	6/8	0/8	6/8	0/8	Stonier *et al*., 2017

*Assessed by enzyme-linked immunosorbent assay

1.10.1.2 Immunofluorescent assays

Initial investigations of filovirus disease outbreaks heavily relied on immunofluorescent assays (IFs) for the detection of anti-filovirus IgG in sera from patients suspected to be infected (Saijo *et al*., 2006a). The first indirect IF method employing polyvalent antigens (mixtures of Vero cells infected with different viruses) to detect antibodies to Lassa virus, MARV and EBOV, was published several decades ago by Johnson and colleagues (1981). This method was reported as being sensitive (Johnson *et al*., 1981), but potential non-specificity noted in human populations with a low risk of infection resulted in a lack of confidence in the assay and restricted its use (Bower & Glyn, 2017). However, ELISA-based studies have since produced similar results (Nakounne *et al*., 1990; Gonzales *et al*., 2000), suggesting that asymptomatic infection or unrecognised exposure to these or other antigenically related viruses might have occurred in these populations. Preparation of antigens for IF by live virus propagation are limited to BSL-4 facilities. However, diagnostic assays for MVD are essential in countries where MARV is endemic but BSL-4 facilities are not available. For this reason, it is crucial that recombinant protein-based diagnostic assays are developed. Immunofluorescent methods employing HeLa cells infected with recombinant baculovirus expressing EBOV NP (Saijo *et al*., 2001b) or RESTV NP (Ikegami *et al*., 2002), have been developed, but no such assay has been described for MARV to date.

1.10.1.3 Enzyme-linked immunosorbent assays

A variety of ELISAs is available for the detection of filovirus-specific antibodies in sera (Table 1.2). Ksiazek and colleagues (1999) described an indirect ELISA (I-ELISA) for filoviruses utilising crude antigens prepared from virus-infected Vero cell lysate, and control antigens prepared from mock-inoculated cells. The I-ELISA was reported to be more specific than classic IF methods (Ksiazek *et al*., 1999). This assay remains used for MARV diagnosis in WHO VHF reference and research laboratories worldwide. While crude antigens are generally easy to prepare in large quantities, the presence of non-specific proteins in the cell lysates may lead to reduced specificity, cross-reactivity and, subsequently, false positive results (Khalil *et al*., 1990; Sobarzo *et al*., 2012). In contrast, making use of recombinant antigens may reduce

the risk of cross-reaction, ease standardisation and eliminate the need for using infectious agents as antigens (Lopez *et al*., 2009).

Prehaud and colleagues first reported the value of recombinant proteins in filovirus diagnostics in 1998. Several other groups have since made use of recombinant proteins in the development of ELISAs for the diagnosis of filoviral disease. Recombinant IgG ELISAs were developed by Saijo and colleagues (2001a) using a full-length NP of EBOV expressed in a baculovirus system, as well as carboxy-terminal halves of the NPs of EBOV and MARV expressed in an *Escherichia coli* (*E. coli*) system, as antigens. The EBOV assay was reported as being highly sensitive and specific for the detection of anti-EBOV antibodies; however, the sensitivity for the MARV ELISA could not be determined due to the unavailability of a large enough panel of MARV antibody-positive sera (Saijo *et al*., 2001a).

Nakayama and colleagues (2010) developed species-specific ELISAs using recombinant Histidine-tagged GPs of the five ebolavirus species and of MARV as antigens. The assays made use of the mammalian expression vector pCAGGS-MCS and human embryonic kidney 293 cells with SV40 large T-antigen (HEK 293T) for the expression of antigens (Nakayama *et al*., 2010). Some cross-reactivity between the GPs from multiple filovirus species was observed, although the greatest reaction of IgG was directed against the GP antigen specific to the species with which the patient was infected (Nakayama *et al*., 2010). The assay was able to detect both IgM and IgG antibodies to MARV in patients infected with the virus (Nakayama *et al*., 2010) and showed promise for use in MVD diagnostics and serosurveillance studies.

1.10.1.4 Virus neutralisation assays

Virus neutralisation tests (VNTs) are regarded as the gold standard for determining the presence of neutralising antibody in serum samples from patients with suspected viral infections. However, VNTs are rarely employed for routine diagnostics and surveillance for MARV as neutralising antibody responses to the virus are either very weak or non-existent in humans, NHPs and bats (Bale *et al*., 2012b; Paweska *et al*., 2012; Flyak *et al*., 2015; Stonier *et al*., 2017). Furthermore, the use of VNTs is restricted to BSL-4 facilities, and these assays are therefore of limited use in countries where MARV is endemic.

Virus neutralisation tests previously used for the detection of antibodies against filoviruses in serum have been reported as being time-consuming and difficult to reproduce (World Health Organisation, 1978a; Spickler, 2010). The use of VNTs is generally restricted to the development of serum panels with conclusive immune status to filoviruses (which is used for the validation of other serological assays such as ELISA), and for use as a confirmatory test. Nevertheless, the VNT remains a useful research tool for characterising the neutralising immune responses of incidental and reservoir hosts against MARV infection.

In-house plaque reduction neutralisation tests (PRNTs) are frequently employed in studies on filovirus-neutralising antibodies and *in vitro* neutralisation. The first PRNT for filoviruses was developed for EBOV and SUDV by Moe and colleagues (1981) (Table 1.2), but the assay was reported to be inconsistent (Truant *et al*., 1983) and less sensitive than indirect fluorescent antibody assays and mouse neutralisation tests. This assay had a further disadvantage in that it took seven days to complete (Moe *et al*., 1981). More recently, pseudovirus neutralisation assays have been used for the detection of neutralising antibodies to filoviruses (Yuan *et al*., 2012; Fusco *et al*., 2015; Yang *et al*., 2017; Zhang *et al*., 2017; Callendret *et al*., 2018). These assays make use of recombinant human immunodeficiency viruses or vesicular stomatitis viruses pseudotyped with filovirus GP and containing either luciferase reporter or green fluorescent protein genes (Fusco *et al*., 2015; Zhang *et al*., 2017). To screen antibodies for *in vitro* neutralisation activity, the pseudoviruses are incubated with test sera and inoculated into cell monolayers, and after a period of incubation, cells are lysed and luciferase activity measured using a luciferase kit and luminometer (Yuan *et al*., 2012), or green fluorescent protein-expressing cells are counted (Fusco *et al*., 2015). Pseudovirus neutralisation assays may be performed without the need for BSL-4 containment, and therefore offers an advantage over traditional VNTs that require the propagation of live virus.

1.10.1.5 Luminex technology multiplex assays

Luminex technology has become available for the detection of antibodies to filoviruses (Laing *et al*., 2016; Ayouba *et al*., 2017). The technology uses purified recombinant viral proteins coupled to fluorescently colour-coded BioPlex carboxylated beads (Laing *et al*., 2016) as

antigens. Luminex-based assays permit screening for antibodies to multiple viruses simultaneously in a single well, and are therefore less time-consuming and reagent-intensive than other serological assays such as VNTs and ELISAs (Ayouba *et al*., 2017). Furthermore, antigens are expressed in mammalian cell culture systems and therefore retain their native structures and glycosylation, facilitating the detection of conformationally-dependent virus-specific antibodies (Laing *et al*., 2016). Ayouba and colleagues (2017) developed and validated an ebolavirus-specific Luminex assay that makes use of nine recombinant proteins (NP, GP and VP40) of four of the five ebolavirus species. The assay was used to screen the sera of 94 survivors of EBOV disease and 108 sera from patients never infected with the virus. Results indicated assay sensitivities of 95.7%, 96.7% and 92.5%, and specificities of 94.4%, 95.4% and 96.3%, respectively for the NP, GP and VP40 proteins of EBOV (Ayouba *et al*., 2017). Compared to commercially available ELISAs based on the NP and GP of EBOV (Alpha Diagnostics), the ELISA assay had a lower sensitivity (92.5% vs 95.7%) than the Luminex assay but a slightly improved specificity (100% vs 99.1% for the Luminex assay) (Ayouba *et al*., 2017). The Luminex assay was also reported to be less expensive than commercial ELISA assays. Similar technology for MARV could prove useful for surveillance and MVD diagnosis.

1.10.1.6 Protein microarrays

Recombinant protein microarrays provide a safe alternative to serological assays that make use of live MARV preparations. Furthermore, these assays allow the detection of antibodies to several viral proteins simultaneously. Kamata and colleagues (2014) described a protein microarray composed of the GP, NP and VP40 of all ebolavirus and marburgvirus species (Table 1.2). The assay was able to detect increases in protein-specific IgM and IgG antibody levels in vaccinated NHPs, however, further optimisation and validation efforts will be required before the test can be applied in MVD diagnosis and serosurveillance.

1.10.2 Marburg virus antigen detection tests

Antigen capture ELISAs are useful during the early stages of infection before convalescence occurs (8 to 10 days after the onset of symptoms) (Rougeron *et al*., 2015). Although antigen capture ELISAs are used widely by WHO reference laboratories in the diagnosis of MVD, these

assays are all in-house and have not been thoroughly described in the literature. Saijo and colleagues (2005; 2006b) produced two clones of monoclonal antibodies to recombinant MARV Musoke NP in immunised mice and reported their efficacy in antigen capture ELISA format. The assay was reported to have a similar sensitivity to the RT-PCR described by Sanchez and colleagues (1999). Sherwood and colleagues (2007) described an antigen capture assay for MARV Musoke, Ravn and Angola that made use of NP-specific llama single domain antibodies. While the assay was not optimised, it was reported to be rapid, sensitive and specific, with no cross-reactivity occurring with other filovirus species.

1.10.3 Molecular techniques for the detection of Marburg virus nucleic acid

Molecular methods are currently the tools of choice for the diagnosis of MVD in WHO VHF reference laboratories, and are also used in MARV surveillance studies in bat populations (Amman *et al*., 2012; Paweska *et al*., 2018). Nucleic acid of MARV can be detected in blood from the third day after the onset of symptoms in humans, and may remain detectable in the blood up to the 16th day after the onset of symptoms (Martines *et al*., 2015). Experimental inoculation studies have shown that molecular methods are able to detect MARV RNA in the blood of bats from 1 to 12 days post-infection (p.i.) (Paweska *et al*., 2012; Amman *et al*., 2015; Paweska *et al*., 2015; Schuh *et al*., 2017a), in oral swabs, rectal swabs and urine specimens from 5 to 19 days p.i. (Amman *et al*., 2015; Schuh *et al*., 2017a) and in the liver, spleen and other tissues from 3 to 28 days p.i. (Paweska *et al*., 2012; Amman *et al*., 2015; Paweska *et al*., 2015). While these assays are important in confirming MARV infection in reservoir host bat populations during surveillance studies, the short period of viraemia and low levels of viral shedding make the detection of actively infected individuals in the wild difficult. Molecular assays should therefore be used in combination with serological assays for MARV surveillance.

As of 2018, the most recently published molecular assays for the detection of filovirus RNA include a consensus RT-PCR assay using a cocktail of primers targeting the L gene of filoviruses (Zhai *et al*., 2007), a qRT-PCR assay using five primers and three probes targeting the L gene of filoviruses (Panning *et al*., 2007), and a conventional RT-PCR assay using four primers targeting the NP gene of MARV and EBOV (Ogawa *et al*., 2011). The assays targeting the L-gene have been shown to be able to detect different strains of EBOV, SUDV, MARV,

TAFV and RESTV with high analytical sensitivities (Panning *et al*., 2007; Zhai *et al*., 2007), while the assay targeting the NP gene was able to detect EBOV, MARV, SUDV, TAFV, RESTV and BDBV (Ogawa *et al*., 2011). A commercial kit (RealStar Filovirus Screen, Altona Diagnostics) based on the qRT-PCR method described by Panning and colleagues (2007) has also become available and has been shown to have a high diagnostic sensitivity with good differentiation between different filovirus species (Rieger *et al*., 2016). The major obstacles in designing an RT-PCR assay for filoviruses include the high genetic diversity between the different filovirus genera, and the inability to determine the clinical sensitivity of the assays due to the unavailability of well-characterised serum panels of patients infected with different filovirus species (Panning *et al*., 2007; Zhai *et al*., 2007).

1.10.4 Virus isolation

Virus isolation in Vero E6 African green monkey kidney cells is the traditional gold standard technique to confirm the presence of MARV in a specimen. Virus isolation allows direct visualisation of MARV by electron microscopy within 1 week post inoculation. Although definitive, virus isolation methods require BSL-4 containment and are therefore restricted to laboratories outside of countries where MARV is endemic (Broadhurst *et al*., 2016).

1.11 Significance and aims of this study

Egyptian rousette bats have been implicated as reservoir hosts for MARV and tools for detecting and monitoring MARV infection in these animals therefore need to be developed or improved. The NICD-NHLS in South Africa is responsible for MARV diagnosis and surveillance in Africa. It is therefore essential that in-house capacity for the production of recombinant ELISA antigens be established. Antibodies against MARV have been detected in African bat species, and serological and molecular surveys have shown that MARV is also present in ERBs in South Africa (Paweska *et al*., 2018). Although no human outbreaks of MVD have occurred in South Africa at the time of writing, the detection of MARV in South African bats is worrying, as this is the first time that evidence of this highly pathogenic virus has been found in a reservoir host species in Southern Africa. The development of improved MARV serological assays specific to bat sera will provide surveillance capacity to monitor the immune

status of reservoir host populations, which will assist in predicting potential spillover events into humans. In addition, these tools will contribute to the characterisation of the immune responses of ERBs to MARV infection and ultimately assist in elucidating the mechanisms by which bats are able to maintain the virus in nature and combat clinical disease.

The aims and objectives of this study were as follows:

- To develop and evaluate ELISAs based on recombinant antigens for the detection of MARV antibodies in ERB sera:
    - To produce antigen using recombinant DNA technology by cloning, expressing and purifying major recombinant MARV antigens (NP and GP);
    - To evaluate the recombinant antigens for suitability as diagnostic reagents in ELISA format;
    - To develop recombinant ELISAs in I-ELISA format;
    - To optimise and evaluate the I-ELISAs for use with ERB serum by determining the repeatability (inter- and intra-plate variation), sensitivity and specificity of each assay.

- To monitor and evaluate the dynamics and characteristics of antibody responses to MARV in ERBs:
    - To apply an I-ELISA developed in this study in the detection of maternal antibodies to MARV in juvenile ERBs;
    - To determine the dynamics of antibody responses in experimentally and naturally infected bats;
    - To determine whether previously naturally infected ERBs can become reinfected with MARV, or whether antibodies are fully protective against reinfection.

CHAPTER 2: DEVELOPMENT AND EVALUATION OF ENZYME-LINKED IMMUNOSORBENT ASSAYS FOR THE DETECTION OF ANTI-MARBURG VIRUS IMMUNOGLOBULIN G ANTIBODIES IN EGYPTIAN ROUSETTE BATS

2.1 Introduction

The Egyptian rousette bat (ERB) is a reservoir host for Marburg virus (MARV) (Swanepoel *et al*., 2007; Towner *et al*., 2007; Towner *et al*., 2009; Paweska *et al*., 2012), which causes a severe haemorrhagic disease in humans and non-human primates (Feldmann *et al*., 2013). Surveillance studies in reservoir host bat populations are essential to determine where the virus is prevalent and where there is a risk of spillover into human and other animal populations. Experimental MARV infection studies have identified a relatively short period of viraemia in ERBs, with viral ribonucleic acid (RNA) only being detectable in the blood and saliva up to 2 weeks post-infection (Paweska *et al*., 2012; Amman *et al*., 2015; Paweska *et al*., 2015; Schuh *et al*., 2017a). The short period of viraemia in MARV-infected bats makes it challenging to detect actively infected individuals in the wild using molecular methods and virus isolation. Furthermore, ecological studies of MARV in ERB populations in Uganda and South Africa have indicated a high seroprevalence for the virus, but a low frequency of virus detection in these bats (Amman *et al*., 2012; Paweska *et al*., 2018). For this reason, accurate serological assays are crucial for filovirus surveillance in wild bat populations. Further to their importance in serosurveillance, serological assays are essential for monitoring the immune status of reservoir host populations and may assist in predicting when large transmission events are imminent.

Serological enzyme-linked immunosorbent assays (ELISA) have been developed for the detection of antibodies to MARV. Ksiazek and colleagues (1999) described an indirect ELISA (I-ELISA) for filoviruses that remains widely used in World Health Organisation (WHO) reference laboratories for the diagnosis and surveillance of viral haemorrhagic fevers (Bausch *et al*., 2006; Swanepoel *et al*., 2007; Towner *et al*., 2007; Pourrut *et al.,* 2009; Paweska *et al*., 2012). The assay detects antibodies to MARV or other filoviruses by a two-step procedure that involves binding of filovirus-specific immunoglobulin G (IgG) in test sera to an antigen, followed by detection of the IgG antibodies by a horseradish peroxidase (HRP)-labelled

secondary antibody. The first generation I-ELISA described by Ksiazek and colleagues (1999) made use of filovirus-infected Vero E6 cell lysate as an antigen. Serological assays for filoviruses based on viral lysates are prone to false positive reactions due to the binding of serum antibodies to cellular contaminants such as the major histocompatibility complex (Rao *et al*., 1997). In addition, preparation of antigens in this manner is limited to biosafety level four (BSL-4) laboratories, which are not available in countries where filoviruses are endemic. For these reasons, second generation I-ELISAs for filoviruses have been developed that make use of recombinant proteins as antigens (Saijo *et al*., 2001a; Nakayama *et al*., 2010; Sobarzo *et al*., 2012). These assays are more specific, sensitive and reproducible, and could be performed without the need for maximum biocontainment.

The Centre for Emerging Zoonotic and Parasitic Diseases (CEZPD) of the National Institute for Communicable Diseases of the National Health Laboratory Service (NICD-NHLS) in South Africa is a WHO Collaborating Centre for Research and Reference on Viral Haemorrhagic Fevers and Arboviruses in Africa. As of 2018, the CEZPD uses an adapted version of the I-ELISA described by Ksiazek and colleagues (1999) that replaces the crude antigens with commercially available MARV glycoprotein (GP) (Integrated BioTherapeutics) for MARV serosurveillance and research (Paweska *et al*., 2015; Paweska *et al*., 2018), but continues to make use of the crude antigen for the diagnosis of MARV disease (MVD). Similarly, several other viral haemorrhagic fever diagnostic and research laboratories now make use of in-house or commercially available recombinant MARV antigens in their ELISAs (Amman *et al*., 2012; Amman *et al*., 2015; Schuh *et al*., 2017a). However, commercially available antigens are expensive, and none of these assays have been validated for use with human or ERB serum, mainly due to the unavailability of large enough serum panels from infected individuals. Methods for producing in-house recombinant protein antigens for MARV-specific ELISAs are currently not available at the CEZPD. Establishment of in-house capacity for the production of these antigens will allow more cost-effective and accurate diagnosis of MVD and will assist in the biosurveillance programme in South Africa aimed at monitoring the presence and distribution of MARV in local bat populations.

Different expression systems have been employed in the production of recombinant MARV proteins. These include bacterial (Saijo *et al*., 2001; Huang *et al*., 2014; Amman *et al*., 2015;

Schuh *et al*., 2017a), baculovirus-insect (Saijo *et al*., 2001) and mammalian systems (Nakayama *et al*., 2010). The selection of an expression system depends on several factors including cost, ease of use, amount of protein required, the importance of post-translational modifications and protein application. Due to their short doubling time, bacteria are rapid, simple and inexpensive systems for expressing recombinant proteins in large amounts. However, bacterial expression systems are incapable of molecular folding, and expressed proteins are therefore biologically inactive (Rosano & Ceccarelli, 2014). Furthermore, bacteria lack the enzymes responsible for eukaryotic post-translational modifications such as attaching sugar residues to GPs (Rosano & Ceccarelli, 2014). Baculoviral expression of proteins in insect host cells overcomes some of the problems presented by bacterial expression systems. However, the protein glycosylation pathways differ between insect and mammalian cells (Jarvis *et al*., 1998), which may considerably affect the antigenic properties of the expressed recombinant protein (Nakayama *et al*., 2010). The mammalian expression system is therefore the most suitable method for expressing appropriate MARV proteins.

Several studies have indicated that the GP (Sobarzo *et al*., 2012; Sobarzo *et al*., 2013; Becquart *et al*., 2014; Stonier *et al*., 2017) and nucleoprotein (NP) (Groen *et al*., 2003; Sobarzo *et al*., 2012; Sobarzo *et al*., 2013; Stonier *et al*., 2017) of filoviruses are the most immunogenic due to their location in the virion and their abundance. These proteins are therefore the most suitable antigens for use in serological assays such as ELISA. The GP is the most genetically diverse filovirus protein, with a 72% difference between filovirus species at the amino acid level (Sanchez *et al*., 1998). This protein is therefore considered to be species-specific and is a valuable antigen for use in serological assays where the filovirus species with which a patient is infected needs to be determined. Enzyme-linked immunosorbent assays based on recombinant NP antigens of Ebola virus (EBOV) have successfully detected NP-specific antibodies in animals infected with EBOV, Sudan virus, Taï Forest virus and Reston virus, demonstrating strong cross-reactivity for this protein between the different ebolavirus species (Saijo *et al*., 2001a). The NPs of the ebola- and marburgviruses have been shown to have comparable amino acid sequences in their N-terminal halves (Sanchez *et al*., 1992) and share at least one conserved antibody epitope in their C-terminal halves (Ali & Islam, 2015). Cross-reactivity may therefore also occur for this protein between the filovirus genera. Consequently,

recombinant NP antigens may be useful in serological screening tests when the specific virus with which an individual is infected, is unknown (Saijo *et al*., 2001a; Nakayama *et al*., 2010).

In this chapter, the development of two I-ELISAs based on recombinant MARV NP or Histidine (His)-tagged GP antigens, expressed in a mammalian expression system, is described. The I-ELISAs were evaluated and their performance characteristics compared by testing sera collected from bats naturally or experimentally infected with MARV. Results from the two assays developed in this study were further compared to results obtained using an I-ELISA based on commercially available MARV GP (Integrated BioTherapeutics). Both assays were found to be robust and repeatable, with good sensitivity and specificity. However, the sensitivity and specificity of the MARV GP-based I-ELISA were higher (98.8% and 100%, respectively) than that of the MARV NP-based I-ELISA (96.3% and 96.9%, respectively). The GP-based I-ELISA showed no cross-reactivity of IgG antibodies in sera from bats experimentally infected with EBOV. The NP-based I-ELISA, however, showed cross-reaction in the sera of three out of five EBOV-infected bats. Marburg virus-specific IgG antibodies could be detected in sera from experimentally infected bats from 5 days post-infection (p.i.) using the GP-based I-ELISA and from 9 days p.i. using the NP-based I-ELISA. These results demonstrate the applicability of the I-ELISAs as tools for diagnosing MARV disease (MVD), characterising reservoir host immune responses, and serosurveillance.

2.2 Materials and methods

2.2.1 Production of recombinant antigens

2.2.1.1 Primer design

The prototype Musoke strain of MARV was chosen for the production of the recombinant antigens for this study. Primers for the cloning of recombinant MARV Musoke proteins were designed from the GP (amino acid position 1 – 636, transmembrane domain removed) and NP (full-length, amino acid position 1 – 695) gene sequences of the MARV reference strain, which are available in the public domain (GenBank, www.ncbi.nlm.noh.gov; accession number NC_001608.3). For ligation of the genes of interest into a pCAGGS-MCS mammalian expression vector (Figure 2.1), an EcoRI restriction site (5' GAA TTC 3') was incorporated into

the MARV GP and NP forward primers and an NheI restriction site (5' GCT AGC 3') was incorporated into the MARV GP and NP reverse primers.

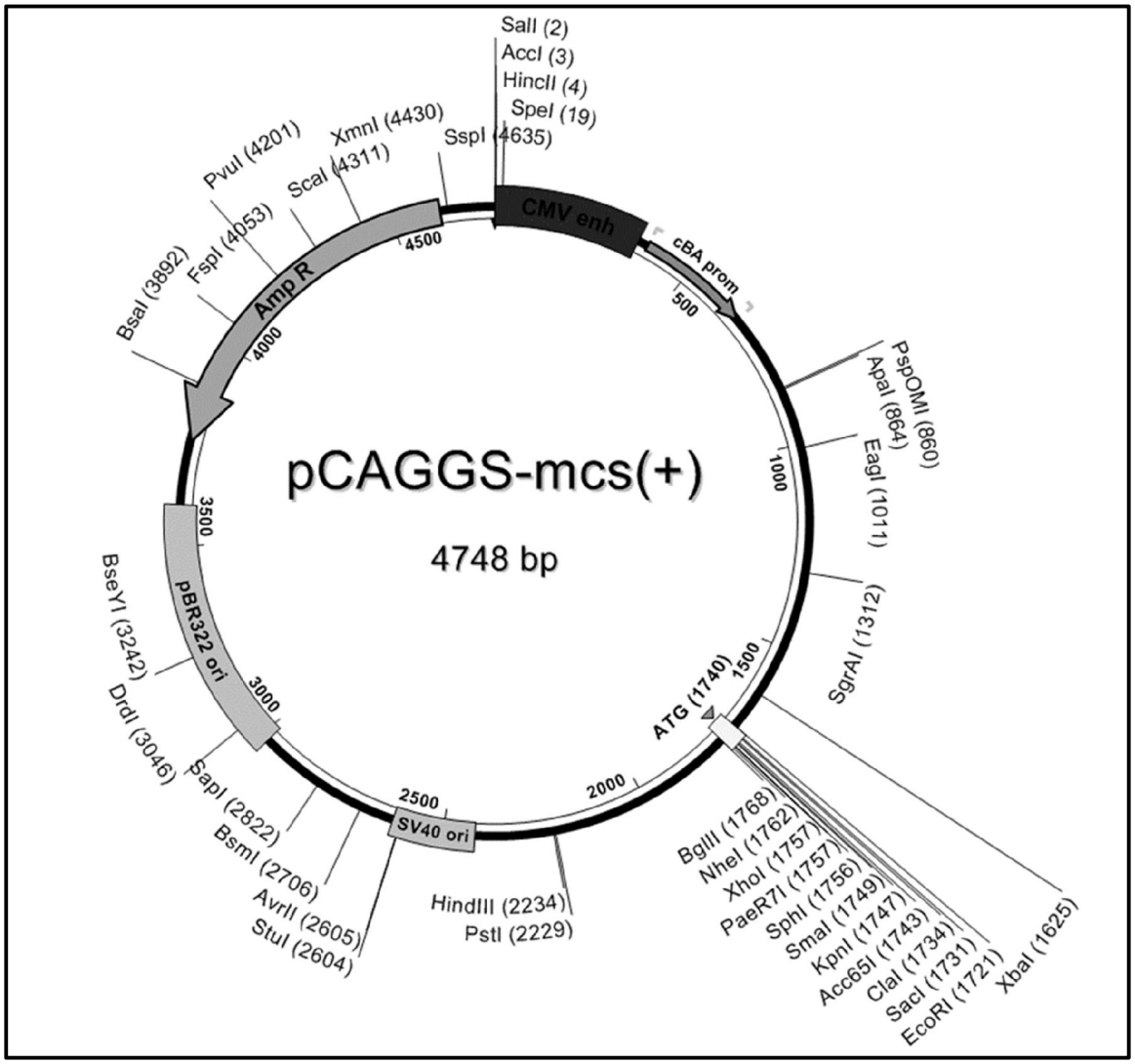


Figure 2.1: Map of pCAGGS-MCS mammalian protein expression vector (4 748 bp) containing a cytomegalovirus enhancer (CMV enh), chicken β-actin promoter (cBA), SV40 origin of replication (ori) and Ampicillin resistance gene (Amp R). Image obtained from Prof Ayato Takada, Centre for Zoonosis Control, Hokkaido University, Sapporo, Japan.

Kozak consensus sequences (5' GCC ACC 3') were added to both the MARV GP and NP forward primers to facilitate translation initiation in mammalian cells. Three additional nucleotides were added to the 5' ends of the MARV NP forward and reverse primers to improve restriction enzyme digestion. These additional nucleotides were not included in the MARV GP primers as they adversely affected the primer melting temperatures. Stop codons (5' TCA 3' or 5' TTA 3') were incorporated into the MARV NP and GP reverse primers. A His-tag (5' ATG ATG ATG ATG ATG ATG 3') was incorporated into the MARV GP reverse primer to facilitate protein purification. The primers were analysed for melting temperature, GC content, hairpins and dimers using OligoAnalyzer 3.1 (Integrated DNA Technologies). The primers are listed in Table 2.1.

Table 2.1: Primers for the cloning of Marburg virus Musoke genes

Marburg virus Musoke gene	Primer name	Sequence (5' – 3')[a]	Position (5' – 3')[b]	Melting temperature	Product size
NP	EcoRI Musoke NP-F	TAC GAA TTC GCC ACC ATG GAT TTA CAC AGT TTG TTG GAG	104-127	64.6°C	2114 bp
	NheI Musoke NP-R	ACA GCT AGC TCA TCA CAA GTT CAT CGC AAC ATG TCT CC	2166-2188	65.5°C	
GP	EcoRI-Musoke GP-F	GAA TTC GCC ACC ATG AAG ACC ACA TGT TTC CTT ATC AGT CTT ATC	5941-5973	64.5°C	1979 bp
	NheI Musoke GP-His-R	GCT AGC TTA TCA ATG ATG ATG ATG ATG ATG TGT CCA CCA TTT ACC ACC CAG ACC CCA	7852-7878	68.7°C	

[a] Purple coloured nucleotides indicate restriction enzyme sites (EcoRI for forward primers and NheI for reverse primers), blue nucleotides indicate Kozak sequences, green nucleotides indicate 6 x Histidine (His) and red nucleotides indicate stop codons

[b] Position numbered according to Marburg virus Musoke reference sequence, GenBank accession number NC_001608.3 (Enterlein *et al*., 2006)

2.2.1.2 Ribonucleic acid extraction

Marburg virus RNA was extracted from the supernatant of Vero E6 cells infected with the Musoke strain using the QIAamp Viral RNA Mini kit (QIAGEN) according to the

manufacturer's instructions. Supernatant from an uninfected control flask of Vero E6 cells was identically processed as a negative control. Briefly, 140 µl of supernatant was added to a microcentrifuge tube with 560 µl AVL lysis buffer containing 5.6 µl carrier RNA (1 µg/µl). The contents of the tube was mixed for 15 seconds and incubated at 25°C for 10 minutes. A volume of 560 µl 96% ethanol (Merck) was added and the contents mixed for 15 seconds. Six hundred and thirty microliters of the solution was added to a QIAamp Mini column in a collection tube. The tube was centrifuged at 8 000 x *g* for 1 minute (Eppendorf MiniSpin, Merck). The filtrate was discarded and the previous step repeated. Five hundred microliters of buffer AW1 was added and the tube centrifuged at 8 000 x *g* for 1 minute (Eppendorf Mini Spin, Merck). After discarding the filtrate, 500 µl of buffer AW2 was added. The tube was centrifuged at 12 100 x *g* for 3 minutes (Eppendorf Mini Spin, Merck). The QIAamp Mini column was placed into a sterile microcentrifuge tube and centrifuged at 12 100 x *g* for 1 minute (Eppendorf Mini Spin, Merck). After placing the column into a clean microcentrifuge tube, 60 µl of buffer AVE was added. The tube was incubated at 25°C for 1 minute, followed by centrifugation at 8 000 x *g* for 1 minute (Eppendorf Mini Spin, Merck). The eluted RNA was stored at -70°C until required.

2.2.1.3 Reverse transcription polymerase chain reaction (cDNA synthesis)

A two-step reverse transcription polymerase chain reaction (RT-PCR) was performed using the Moloney murine leukaemia virus (M-MLV) reverse transcriptase kit (Invitrogen) as follows: 8 µl of MARV Musoke RNA or negative control eluate (see section 2.2.1.2) and 2 µl gene-specific forward primer (10 µM; Sigma-Aldrich) (Table 2.1) was added to a PCR tube. The tubes were incubated in a thermocycler (2720 Thermal Cycler, Applied Biosystems) at 70°C for 5 minutes and cooled at 4°C. Eight microlitres of 5 x buffer (250 mM Tris hydrochloride (Tris-HCl), 375 mM potassium chloride (KCl) and 15 mM magnesium chloride ($MgCl_2$)), 4 µl dithiothreitol (DTT) (0.1 M), 1 µl RNase inhibitor (40 units/µl), 1 µl M-MLV reverse transcriptase (200 units/µl), 2 µl deoxyribonucleotide triphosphate (dNTP, 10 mM) and 14 µl nuclease-free water was added to the control and template tubes (final volume: 40 µl). The PCR tubes were incubated in a thermocycler (2720 Thermal Cycler, Applied Biosystems) at 42°C for 1 hour and 98°C for 5 minutes. The MARV complementary DNA (cDNA) and negative control PCR product were stored at 4°C until required.

2.2.1.4 Amplification of Marburg virus Musoke glycoprotein and nucleoprotein genes

The MARV Musoke GP and NP gene fragments were amplified using the KOD-Plus-Neo kit (Toyobo) as follows: 5 µl of 10 x KOD PCR buffer, 5 µl of 2 mM dNTPs, 3 µl of 25 mM magnesium sulphate ($MgSO_4$), 1.5 µl of each primer (forward and reverse) (10 µM; Sigma-Aldrich), 1 µl KOD-Plus-Neo enzyme (1 unit/µl), 2 µl template cDNA, negative control PCR product from section 2.2.1.3 or positive control DNA template (Zero Blunt TOPO PCR cloning kit; Invitrogen) and 31 µl nuclease-free water was added to a PCR tube (total reaction volume: 50 µl). Primers for the amplification of NP and GP genes are described in Table 2.1. The same primers were used for the negative control reactions. To amplify the positive control DNA template, M13 forward (5' GTA AAC GAC GGC CAG 3') and reverse (5' GTC ATA GCT GTT TCC TG 3') primers were used. For amplification of the NP gene and positive control DNA template, the cycling conditions were set according to the manufacturer's instructions, with a pre-denaturation step at 94°C for 2 minutes, followed by 40 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 30 seconds and extension at 68°C for 30 seconds. A two-step protocol was followed for the amplification of the His-tagged GP gene as follows: pre-denaturation at 94°C for 2 minutes, followed by 40 cycles of denaturation at 98°C for 10 seconds and extension at 68°C.

2.2.1.5 Analysis and purification of amplification products

The amplification products were analysed using agarose gel electrophoresis as described by Sambrook and Russell (2001). Briefly, 5 µl of template or control and 1 µl blue/orange 6 x loading dye (Promega) was loaded onto a 0.8% (m/v) agarose (Seakem LE) gel containing 1 x GelRed (Biotium). A 1 000 bp molecular weight marker (Nippon Genetics) was included as a reference for each gel. The samples were electrophoresed (Power Pac 300, BioRad) for 40 minutes at 110 V. The amplicons of the RT-PCR reactions were visualised using an ultraviolet light box (White/UV Transilluminator, UVP). The expected band sizes were approximately 1 980 bp for the GP gene (Table 2.1), 2 100 bp for the NP gene (Table 2.1) and 800 bp for the positive control.

The PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Briefly, 45 µl of membrane binding solution was added to the PCR amplicons. An SV minicolumn was positioned in a collection tube for each PCR product. The prepared PCR product was transferred to the minicolumn assembly and incubated at 25°C for 1 minute, followed by centrifugation (Zentrifugen Mikro 200, Hettich) at 14 000 x *g* for 1 minute. The filtrate was discarded, the minicolumn was returned to the collection tube, and 700 µl of membrane wash solution (diluted with 95% ethanol (Merck)) was added. The SV minicolumn assembly was centrifuged (Zentrifugen Mikro 200, Hettich) for 1 minute at 14 000 x *g*. The collection tube was emptied, and the wash was repeated with 500 µl of membrane wash solution. Centrifugation was performed for 5 minutes at 14 000 x *g* (Zentrifugen Mikro 200, Hettich). The SV minicolumn was transferred to a new, sterile 1.5 ml microcentrifuge tube. Fifty microlitres of nuclease-free water was added, and the column assembly was incubated at 25°C for 1 minute. The column assembly was centrifuged (Zentrifugen Mikro 200, Hettich) at 14 000 x *g* for 1 minute to elute the purified DNA. The concentration of the DNA was calculated by means of spectrophotometry using the Nanodrop 1 000 (Thermo Fisher Scientific). Briefly, after making a blank measurement with 1 µl nuclease-free water, 1 µl of purified DNA was pipetted onto the measurement pedestal of the Nanodrop 1 000. The sampling arm was closed, and a spectral measurement was initiated using the nucleic acid application module of the system operating software, which measures the absorbance of the sample at 260 nanometers. The DNA was stored at -20°C until required for further processing.

2.2.1.6 Cloning of the Marburg virus glycoprotein and nucleoprotein genes into the pCR-Blunt II-TOPO vector

To facilitate easy sequencing and ensure efficient restriction enzyme digestion, the GP and NP genes of MARV Musoke, and positive control DNA were cloned into a TOPO vector using the Zero Blunt TOPO PCR cloning kit (Invitrogen) according to the manufacturer's instructions. Briefly, 3 µl of purified PCR product (GP DNA: 58.3 ng/µl; NP DNA: 126.5 ng/µl; positive control DNA: 26.9 ng/µl) or nuclease-free water (vector-only control), 1 µl of salt solution (1.2 M sodium chloride and 0.06 M magnesium chloride), 1 µl of nuclease-free water and 1 µl pCR-Blunt II-TOPO vector (Figure 2.2) solution (10 ng/µl plasmid DNA in 50% glycerol,

50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM DTT, 0.1% Triton X-100, 100 µg/ml bovine serum albumin and 30 µM bromophenol blue) was added to a sterile tube. The reaction was mixed lightly, incubated at 25°C for 30 minutes, and placed on ice. Two microlitres of each TOPO cloning reaction was added to separate vials (50 µl) of One Shot TOP 10 chemically competent *Escherichia coli* (*E. coli*) cells and mixed gently. As a transformation control, an additional vial of *E. coli* was transformed using 10 picograms of pUC19 vector (Invitrogen). The cells were incubated on ice for 20 minutes, and heat shocked at 42°C for 30 seconds. The tubes were immediately transferred to ice, and 250 µl super optimal broth with catabolite repression (SOC) medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) was added. The tubes were placed horizontally in a shaking incubator (Labcon, VacTech) at 200 revolutions per minute (rpm) at 37°C for 1 hour. The contents of the tubes were spread onto pre-warmed Luria Bertani (LB) agar plates (1% tryptone, 1% NaCl, 0.5% yeast extract, pH 7.4) (Merck) containing 50 µg/ml Kanamycin (Invitrogen) or 100 µg/ml Ampicillin (Invitrogen) (pUC19 transformation control) and incubated overnight at 37°C. As a negative control, untransformed *E. coli* was plated onto LB agar containing 50 µg/ml Kanamycin and incubated overnight at 37°C.

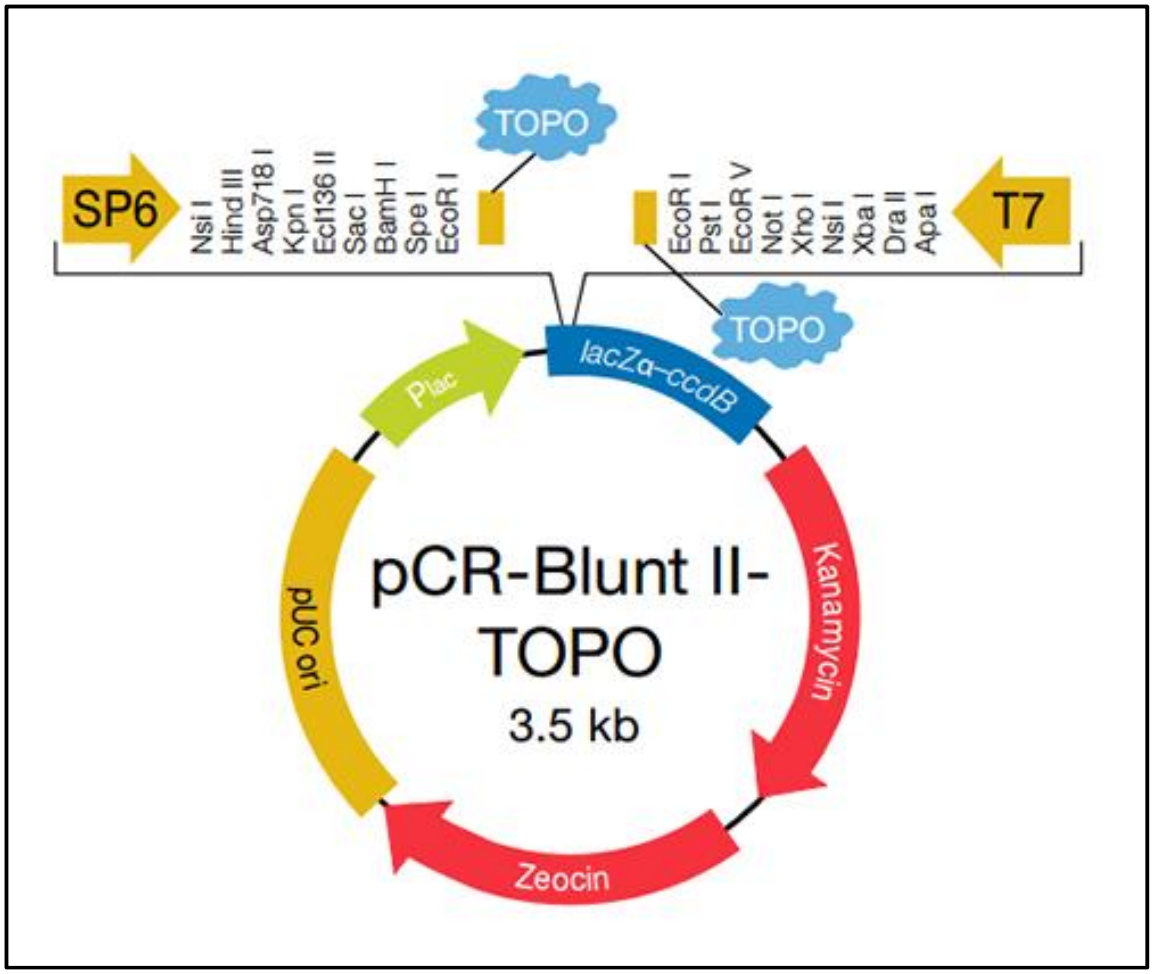


Figure 2.2: Map of pCR-Blunt II-TOPO vector (3.5 kb) containing a T7 promoter, pUC origin of replication, and Kanamycin and Zeocin resistance genes. The yellow bars next to the EcoRI restriction sites in the top panel highlight the insertion site for the blunt PCR product. Image obtained from Invitrogen (https://tools.thermofisher.com/content/sfs/vectors/pcrbluntiitopo_map.pdf).

2.2.1.7 Confirmation of clones by PCR and sequencing

Eight colonies were selected per plate. The inserts of the cloning vectors were confirmed using the GoTaq PCR kit (Promega) as follows: a master mix was prepared for eight reactions using 16 µl of 5 x buffer, 1.5 µl of dNTPs (10 mM), 1 µl of M13 forward primer (20 µM, Invitrogen), 1 µl of M13 reverse primer (20 µM, Invitrogen), 0.5 µl Taq polymerase (5 units/µl) and 60 µl

nuclease-free water. Ten microlitres of master mix was added to each PCR reaction tube. A pipette tip was touched to the selected colony, and the bacteria were mixed into the reaction mix. The tubes were placed in a thermocycler (2720 Thermal Cycler, Applied Biosystems). Cycling conditions were set to 95°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds, 50°C for 60 seconds and 72°C for 150 seconds, and a final extension step at 72°C for 7 minutes. The PCR products were analysed and purified as described in section 2.2.1.4. The concentration of the purified DNA was determined by means of spectrophotometry using the Nanodrop 1 000 (Thermo Fisher Scientific) as previously described (section 2.2.1.5).

Sequencing of the DNA from colonies with the correct size insert (as determined by agarose gel electrophoresis) was performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Two tubes were prepared for each sample by adding 1 µl of sequencing buffer, 1 µl primer (M13 forward or reverse primer, 1.6 µM), 2 µl BigDye v3.1 mix, 5 µl nuclease-free water and 1 µl template DNA (approximately 30 ng/µl) to each tube. The tubes were placed in a thermal cycler (2720 Thermal Cycler, Applied Biosystems) and cycling conditions were set to the following: 94°C for 1 minute, followed by 25 cycles of 94°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. One microlitre of 125 mM EDTA (Merck), 1 µl of 3 M sodium acetate (pH 5.2) (Merck) and 25 µl of 100% ethanol (Merck) was added to each tube. The tubes were shaken and incubated at 25°C in the dark for 15 minutes. The samples were centrifuged (Eppendorf MiniSpin, Merck) for 30 minutes at 12 100 x *g*. The supernatant was removed by pipetting, and 100 µl of 70% ethanol (Merck) was added. The samples were centrifuged (Eppendorf MiniSpin, Merck) for 15 minutes at 12 100 x *g* and the supernatant was removed. Another volume (100 µl) of 70% ethanol (Merck) was added, and the samples were centrifuged (Eppendorf MiniSpin, Merck) at 12 100 x *g* for 15 minutes. The supernatant was removed, and the samples were air-dried in the dark. Twenty microlitres of Hi-Di formamide (Applied Biosystems) was added to each tube, followed by denaturation in a thermal cycler (2720 Thermal Cycler, Applied Biosystems) at 95°C for 5 minutes. Each sequence preparation was added to a 96-well optical reaction plate (Applied Biosystems), and the plate was placed in an ABI 3130 Genetic Analyzer (Applied Biosystems) for sequencing.

Sequences were analysed using the BioEdit Sequence Alignment Editor Version 7 (Hall, 1999), and compared to the MARV Musoke reference strain, accession number NC_001608.3

(Enterlein *et al*., 2006), available in the public domain (GenBank, www.ncbi.nlm.noh.gov). Colonies containing TOPO vectors with identical sequence inserts to the MARV Musoke reference strain in the correct orientation were selected from the LB agar plates and cultured individually in 50 ml centrifuge tubes (NEST) containing 5 ml LB broth (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.4) (Merck) with 50 µg/ml Kanamycin (50 mg/ml stock concentration, Invitrogen). The tubes were placed horizontally in a shaking incubator (200 rpm; Labcon, VacTech) and incubated at 37°C overnight.

2.2.1.8 Plasmid DNA purification

The Wizard Plus SV Minipreps DNA Purification System (Promega) was used to purify plasmid DNA from the overnight cultures. Three millilitres of overnight culture (section 2.2.1.7) was pelleted by centrifugation (Zentrifugen Mikro 200, Hettich) at 10 000 x *g* for 5 minutes. The pellet was resuspended in 250 µl cell resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 100 ug/ml RNase A). Two hundred and fifty microlitres of cell lysis solution (0.2 M NaOH, 1% SDS) was added to each sample, and the tubes were inverted four times to mix the reagents. Ten microlitres of alkaline protease solution (25 µg/µl) was added to each sample, and the tubes were inverted four times to mix the reagents.

The tubes were incubated at 25°C for 5 minutes. Three hundred and fifty microlitres of neutralising solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, pH 4.2) was added to each sample, and the tubes were inverted four times to mix the reagents. The tubes were centrifuged (Zentrifugen Mikro 200, Hettich) at 14 000 x *g* for 10 minutes. A spin column was inserted into a collection tube for each reaction, and 850 µl of cleared lysate was added. The spin columns were centrifuged (Zentrifugen Mikro 200, Hettich) at 14 000 x *g* for 1 minute and the filtrate was discarded. Seven hundred and fifty microlitres of column wash solution (60% ethanol, 60 mM potassium acetate, 8.3 mM Tris-HCl, pH 7.5, 0.04 mM EDTA, pH 8.0) was added, and the spin columns were centrifuged (Zentrifugen Mikro 200, Hettich) at 14 000 x *g* for 1 minute. The filtrate was discarded, 250 µl of column wash solution was added, and the spin columns were centrifuged (Zentrifugen Mikro 200, Hettich) at 14 000 x *g* for 2 minutes. The spin columns were transferred to new sterile 1.5 ml Eppendorf tubes and 100 µl of nuclease-free water was added. The tubes were

centrifuged (Zentrifugen Mikro 200, Hettich) at 14 000 x *g* for 1 minute. The DNA concentration was measured by means of spectrophotometry using the Nanodrop 1 000 (Thermo Fisher Scientific) as previously described. Plasmid isolation was verified by performing agarose gel electrophoresis as described in section 2.2.1.5. Expected band sizes were 5 498 bp for the TOPO-MARV GP plasmid and 5 633 bp for the TOPO-MARV NP plasmid.

2.2.1.9 Restriction enzyme digestion

The constructed plasmids (TOPO-MARV GP 176.6 ng/µl; TOPO-MARV NP 195.3 ng/µl) (section 2.2.1.8) and pCAGGS-MCS expression vector (56.7 ng/µl) (kindly donated by Professor Ayato Takada, Centre for Zoonosis Control, Hokkaido University, Japan) were subjected to restriction enzyme digestion. Briefly, 2 µl of NEBuffer 2.1 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9) (New England Biolabs), 16 µl plasmid DNA or 4 µl pCAGGS-MCS expression vector and 12 µl nuclease-free water, and 1 µl of each restriction enzyme (EcoRI (20 000 units/ml) and NheI (10 000 units/ml); New England Biolabs) was added to a PCR tube. The tubes were incubated in a thermal cycler (2720 Thermal Cycler, Applied Biosystems) at 37°C for 1 hour. Agarose gel electrophoresis was performed by loading the contents of the tubes alongside a 1 000 bp marker (Nippon Genetics), and the bands of the correct sizes (GP insert – 1 979 bp, NP insert – 2 114 bp, pCAGGS backbone – 4 707 bp) were cut out of the gel. The DNA was purified from the gel using the Wizard SV Gel and PCR Clean-Up System (Promega) as described in section 2.2.1.4. The DNA concentration was measured by means of spectrophotometry using the Nanodrop 1 000 (Thermo Fisher Scientific) as described previously (section 2.2.1.5).

2.2.1.10 Sub-cloning of Marburg virus glycoprotein and nucleoprotein genes into the pCAGGS-MCS expression vector

After restriction enzyme digestion and purification, the inserts (section 2.2.1.9) were ligated into the pCAGGS-MCS expression vector as follows: 3 µl of GP or NP insert (~20 ng/µl) or nuclease-free water (negative control), 2 µl of the pCAGGS-MCS expression vector (diluted 10 x) (6.1 ng/µl) and 5 µl of solution I of the DNA ligation kit version 2.1 (T4 DNA ligase in

reaction buffer; contents not specified by manufacturer) (Takara) was added to a microcentrifuge tube and mixed well. The tubes were placed in a cooling block at 16°C for 30 minutes. The ligation reactions were added to microcentrifuge tubes containing 100 µl of DH5α *E. coli* (Life Technologies) and incubated on ice for 20 minutes. In addition, DH5α were transformed with uncut plasmid as a vector-only control. The *E. coli* were heat-shocked in a hot water bath at 42°C for 30 seconds and placed on ice. Two hundred and fifty microlitres of SOC medium was added to each tube. The tubes were placed horizontally in a shaking incubator (200 rpm; Labcon, VacTech) and incubated for 1 hour at 37°C. The transformed *E. coli*, as well as untransformed *E. coli* (negative control), were plated onto LB agar containing 100 µg/ml Ampicillin (100 mg/ml stock concentration; Invitrogen) and incubated at 37°C overnight.

2.2.1.11 Confirmation of cloning into pCAGGS-MCS

Cloning of the inserts into the pCAGGS-MCS vector in the correct orientation was confirmed by conventional PCR and sequencing as described in section 2.2.1.7, except that pCAGGS plasmid-specific forward (5' TGC CTT CTT CTT TTT CCT AC 3') and reverse (5' ATT AGC CAG AAG TCA GAT GC 3') primers (1.6 µM; Inqaba) were used instead of M13 primers. Transformed colonies, containing plasmids with inserts with identical sequences to the MARV Musoke reference strain NC_001608.3 (Enterlein *et al*., 2006), were selected and cultured in 200 ml LB broth containing 100 µg/ml Ampicillin (Invitrogen) in a shaking incubator (200 rpm; Labcon, VacTech) at 37°C overnight.

2.2.1.12 Purification of expression plasmids

Expression plasmids containing the desired insert were purified from the overnight culture in LB broth using the High Purity Plasmid Maxiprep system (Origene) according to the manufacturer's instructions. Briefly, a maxiprep column was equilibrated using 30 ml of equilibration buffer (600 mM NaCl, 100 mM sodium acetate (pH 5.0), 0.15% Triton X-100 (v/v)). The solution was allowed to drain by gravity flow. Two hundred millilitres of overnight culture was pelleted by centrifugation (Allegra X-12R, Beckman Coulter) at 2 380 x *g* for 30 minutes. All medium was removed thoroughly. The cells were resuspended in 10 ml of cell

suspension buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA). Ten millilitres of cell lysis solution (200 mM NaOH, 1% SDS (w/v)) was added, and the tube was inverted five times to mix the contents. The tube was incubated at 25°C for 5 minutes. Ten millilitres of neutralisation buffer (3.1 M potassium acetate (pH 5.5)) was added, and the contents of the tube were mixed by inverting until the solution was homogenous. The mixture was centrifuged at 2 380 x *g* for 10 minutes. The supernatant was applied to the equilibrated column, and the solution was allowed to drain by gravity flow. The column was washed with 60 ml wash buffer (800 mM NaCl, 100 mM sodium acetate (pH 5.0)), and allowed to drain by gravity flow. Fifteen millilitres of elution buffer (1.25 M NaCl, 100 mM Tris-HCl (pH 8.5)) was added to the column and allowed to drain by gravity flow to elute the DNA. The eluted DNA was collected in a 50 ml centrifuge tube (NEST) and 10.5 ml isopropanol (Sigma-Aldrich) was added and mixed. The solution was centrifuged (Allegra X-12R, Beckman Coulter) at 2 380 x *g* for 30 minutes at 4°C. The supernatant was discarded, and 5 ml of 70% ethanol (Sigma-Aldrich) was added. The solution was centrifuged at 2 380 x *g* for 5 minutes at 4°C. The ethanol was discarded, and the pellet was air-dried for 10 minutes. The pelleted DNA was resuspended in 500 µl nuclease-free water, and the concentration was measured by means of spectrophotometry using the Nanodrop 1 000 (Thermo Fisher Scientific) as previously described (section 2.2.1.5). Plasmid isolation was verified by performing agarose gel electrophoresis as previously described (section 2.2.1.5). Expected band sizes were 6 727 bp for pCAGGS-MARV GP, 6 862 bp for pCAGGS-MARV NP and 4 748 bp for pCAGGS without insert. A stock solution with a concentration of 1 mg/ml was prepared from the DNA and was stored at -20°C until further use.

2.2.2 Expression and purification of recombinant Histidine-tagged Marburg virus glycoprotein

2.2.2.1 Cultivation of human embryonic kidney 293T cells

A 90% confluent 75 cm^2 flask of human embryonic kidney 293 cells with SV40 large T-antigen (HEK 293T) (ATCC) was sub-cultured as follows: a sterile aspirator was used to remove medium from cells. The cells were washed once with 10 ml DPBS (Lonza) (pH 7.2). The DPBS was aspirated and 2 ml trypsin-EDTA (Highveld Biologicals) was added. The cells were incubated (Forma Series II, Thermo Fisher Scientific) at 37°C for 2 minutes. Ten millilitres of

Dulbecco's Modified Eagle Medium (DMEM) containing 1% L-glutamine (200 mM solution in 0.85% sodium chloride; Lonza) and 1% antibiotics (penicillin, streptomycin and amphotericin B) (100x concentration; Lonza) was added and mixed lightly using a 10 ml pipette. The cells were transferred to a 50 ml centrifuge tube and centrifuged (Allegra X-12R, Beckman Coulter) at 200 x *g* for 5 minutes. The supernatant was removed and the cells resuspended in 10 ml DMEM. The cells were counted by mixing 25 µl of 0.5% Trypan Blue (Sigma-Aldrich) in DPBS with 25 µl of the cell suspension and applying the mixture to a haemocytometer (Neubauer). Two millilitres of the cell suspension (2 x 10^6 cells/ml) was added to 18 ml DMEM supplemented with 10% irradiated FBS (Hyclone) and transferred to a 75 cm^2 tissue culture flask (NEST). Four additional flasks of cells were prepared in the same manner. The cells were incubated (Forma Series II, Thermo Fisher Scientific) at 37°C in a 5% CO_2 atmosphere overnight.

2.2.2.2 Transfection of cells with Marburg virus GP expression plasmids

Transfection reagent was prepared by adding 15 µg of pCAGGS-MARV GP (1 mg/ml; section 2.2.1.12) or pCAGGS-MCS (1 mg/ml, negative control; section 2.2.1.12) and 60 µl linear 25 kilodalton (kDa) polyethylenimine (1 mg/ml; Sigma-Aldrich) to 600 µl Hank's balanced salt solution without calcium and magnesium (GIBCO, Life Technologies) and mixing well. The solution was incubated at room temperature for 30 minutes. The transfection mixture was gently added to the HEK 293T cells at 50% confluency. The cells were incubated (Forma Series II, Thermo Fisher Scientific) at 37°C in a 5% CO_2 atmosphere for 48 hours.

2.2.2.3 Nickel nitrilotriacetic acid purification

The supernatant was collected from the five flasks of pCAGGS-MARV GP-transfected cells after 48 hours of incubation. Supernatant from cells transfected with pCAGGS-MCS was collected and processed in the same manner as a control. The supernatant was centrifuged (Avanti J-E, Beckman Coulter) at 12 000 x *g* for 10 minutes at 4°C and filtered through a vacuum filter system (pore size 0.45 µM; Corning). Nickel nitrilotriacetic acid (Ni-NTA) agarose resin (Invitrogen) was prepared by mixing well and adding 1.5 ml of the resin to 50 ml centrifuge tubes (NEST). Five millilitres of deionised water was added, and the tubes were

centrifuged (Allegra X-12R, Beckman Coulter) at 800 x *g* for 2 minutes at 4°C and slow acceleration/deceleration. The water was removed, and 5 ml of 1 x Native Purification Buffer (250 mM NaH_2PO_4, pH 8.0; 2.5 M NaCl) (Invitrogen) was added. The tubes were centrifuged (Allegra X-12R, Beckman Coulter) at 800 x *g* for 2 minutes at 4°C and slow acceleration/deceleration. The 1 x Native Purification Buffer and centrifugation step was repeated. The supernatant was removed and discarded. The pH of the MARV GP supernatant was adjusted to 8.0 using either 1 M NaOH (Merck) or 1 M HCl (Merck). Fifty millilitres of MARV GP supernatant was added to the prepared Ni-NTA resin in each tube, and the tubes were placed in a tube rotator (TAITEC RT-5) at 4°C overnight. The tubes were centrifuged (Allegra X-12R, Beckman Coulter) at 800 x *g* for 2 minutes at 4°C and slow acceleration/deceleration. Approximately 45 ml of supernatant was removed into a sterile container, taking care not to disturb the resin. The resin and remaining supernatant was transferred to an Econo-Column 2.5 cm x 10 cm chromatography column (Bio-Rad) and the eluate collected into a sterile container by allowing the column to drain by gravity flow. The eluate was stored at 4°C until required.

Wash buffer was prepared by adding 2.5 ml imidazole (3 M, pH 6.0) (Sigma-Aldrich) to 500 ml 1 x Native Purification Buffer. The pH was adjusted to 8.0 using 1 M NaOH (Merck) or 1 M HCl (Merck). The buffer was filtered through a vacuum filter system (pore size 0.45 µM; Corning). Thirty millilitres of wash buffer was added to the resin in the drained chromatography column and allowed to drain into a 50 ml centrifuge tube (NEST) by gravity flow at 4°C. The tube containing the wash step eluate was stored at 4°C until required. Elution buffer was prepared by adding 25 ml imidazole (3 M, pH 6.0) (Sigma-Aldrich) to 300 ml 1 x Native Purification Buffer. The pH was adjusted to 8.0 and the buffer filtered through a vacuum filter system (pore size 0.45 µM; Corning). Three mini protease inhibitor cocktail tablets (Roche) were added to the elution buffer. After all wash buffer had drained from the chromatography column, 30 ml elution buffer was added to the resin and the eluate collected in fractions of 6 ml each into 15 ml centrifuge tubes. The fractions were stored at 4°C until required for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting.

2.2.2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

An 8% resolving gel was prepared by mixing 4.6 ml deionised water, 2.7 ml of a 30% (w/v) acrylamide/bis-acrylamide solution (Sigma-Aldrich), 2.5 ml Tris-HCl (1.5 M, pH 8.8) (Bio-Rad), 0.1 ml of a 10% SDS solution (Sigma-Aldrich), 0.1 ml of a 10% ammonium persulfate solution (Sigma-Aldrich) and 6 µl tetramethylethylenediamine (TEMED) (Thermo Fisher Scientific). The mixture was poured into an SDS-PAGE cassette (Bio-Rad) and allowed to set. A stacking gel was prepared by mixing 1.72 ml of deionised water, 0.5 ml of a 30% (w/v) acrylamide/bis-acrylamide solution (Sigma-Aldrich), 0.76 ml Tris-HCl (0.5 M, pH 6.8) (Bio-Rad), 30 µl of a 10% SDS solution (Sigma-Aldrich), 30 µl of a 10% ammonium persulfate solution (Sigma-Aldrich) and 3 µl TEMED (Thermo Fisher Scientific). The stacking gel mixture was added on top of the resolving gel, a comb was inserted, and the gel was allowed to set. The SDS-PAGE samples, including the supernatant, wash eluate fractions and elution fractions from section 2.2.2.3 (MARV GP and pCAGGS control) were prepared by mixing 15 µl of sample with 15 µl of 2 x Laemmli sample buffer containing β-mercaptoethanol (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) (Bio-Rad). The samples were incubated in a heating block (Digital Dry Bath, Labnet International, Inc.) at 95°C for 5 minutes. The samples, together with a chemiluminescent protein marker (WesternSure; LiCor) were loaded onto the SDS-PAGE gel. The SDS-PAGE was performed in duplicate to facilitate Coomassie staining and western blotting. The gel tank was filled with SDS-PAGE tank buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) (Bio-Rad) and connected to a PowerPac (Bio-Rad). Electrophoresis was performed at 125 V for 1 hour.

2.2.2.5 Western blotting and Coomassie staining

An SDS-PAGE gel from section 2.2.2.4 was removed from the cassette, and the stacking gel was removed. The gel was covered with InstantBlue Coomassie protein stain (Expedeon) and placed on a shaker (Orbital Shaker, Optic Ivymen System) for 1 hour. The stain was removed, and bands were visualised by eye. The expected band sizes for the GP were approximately 150 kDa (GP_1) and 40 kDa (GP_2 without the transmembrane domain (5 kDa) and with a His-tag (1 kDa)).

A polyvinylidene difluoride blotting membrane (Invitrogen) was soaked in 99.8% methanol (Sigma-Aldrich) and placed on a shaker (Orbital Shaker, Optic Ivymen System) for 1 minute. The methanol was discarded, and western transfer buffer (70 ml deionised water, 20 ml 99.8% methanol and 10 ml 10 x Tris-Glycine buffer) (Novex, Life Technologies) was added to the membrane. The membrane was placed on a shaker (Orbital Shaker, Optic Ivymen System) for 10 minutes. The second SDS-PAGE gel from section 2.2.2.4 was removed from the cassette, and the stacking gel was removed. The resolving gel was placed in a container, and western transfer buffer was added to the gel. The gel was placed on a shaker (Orbital Shaker, Optic Ivymen System) for 10 minutes. The western blotting membrane was placed under the gel, and thick blotting paper (Bio-Rad) was placed under the membrane and on top of the gel. The membrane-gel sandwich was placed on a Trans-Blot SD semi-dry transfer cell (Bio-Rad). The transfer cell was connected to a PowerPac (Bio-Rad), and western blotting was performed at 25 V for 25 minutes.

A blocking buffer was prepared by dissolving 3 g of skim milk powder (Sigma-Aldrich) in 30 ml of 0.05% DPBS containing 10% Tween-20 (PBS-T) (Sigma-Aldrich). The blotting paper and the gel were removed from the membrane, and the membrane was transferred to a container with PBS-T. The container was placed on a shaker (Orbital Shaker, Optic Ivymen System) for 5 minutes. The PBS-T was poured off, and the blocking buffer was added to the membrane. The membrane was placed on a shaker (Orbital Shaker, Optic Ivymen System) for 1 hour. The blocking buffer was poured off, and the membrane washed by adding PBS-T and placing the membrane on a shaker (Orbital Shaker, Optic Ivymen System) for 5 minutes. The PBS-T was poured off. A 1:2 000 dilution of polyclonal rabbit anti-MARV (in-house) was prepared in 1% skim milk in PBS-T and added to the membrane. The membrane was placed on a shaker (Orbital Shaker, Optic Ivymen System) for 1 hour. The antibody was poured off, and the membrane was washed three times with PBS-T, placing the membrane on a shaker (Orbital Shaker, Optic Ivymen System) for 5 minutes between each wash. The PBS-T was discarded. A 1:2 000 dilution of goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (1 mg/ml, Novex, Life Technologies) in 1% skim milk in PBS-T was added to the membrane. The membrane was placed on a shaker (Orbital Shaker, Optic Ivymen System) for 1 hour. The conjugate was poured off, and the membrane was washed three times with PBS-T, placing the membrane on a shaker (Orbital Shaker, Optic Ivymen System) for 5 minutes between each wash. The PBS-T was

poured off, and the membrane was placed on a transparent film. Immobilon western chemiluminescent HRP substrate (Merck) was added to the membrane. Bands were visualised using a ChemiDoc imaging system (Bio-Rad).

2.2.2.6 Dialysis and ultrafiltration

Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher Scientific) were used to perform dialysis on the fractions containing MARV GP (identified in section 2.2.2.5). The cassette membranes were wet using DPBS (Sigma-Aldrich). The fractions containing the correct proteins were injected into the membranes using a syringe, and excess air was removed. Membranes were secured in a float, placed on 5 L of DPBS (Sigma-Aldrich) and placed on a magnetic stirrer (Monostir, Rodwell Scientific Instruments Ltd) at 4°C overnight. The sample was aspirated from the membrane using a syringe, and the protein concentration was measured using the Nanodrop 1 000 (Thermo Fisher Scientific). Briefly, after making a blank measurement with 1 µl DPBS, 1 µl of dialysed protein solution was pipetted onto the measurement pedestal of the Nanodrop 1 000. The sampling arm was closed, and a spectral measurement was initiated using the protein A280 application module of the system operating software, which measures the absorbance of the sample at 280 nanometers.

Ultrafiltration was performed using Amicon Ultra-15 centrifugal filters (Merck). The dialysis sample was transferred to the ultrafiltration tube and centrifuged (Allegra X-12R, Beckman Coulter) at 2 380 x *g* for 10 minutes. Six millilitres of DPBS was added to the ultrafiltration tube and centrifuged (Allegra X-12R, Beckman Coulter) at 2 380 x *g* for 10 minutes at a time until 500 µl of the sample remained in the filter membrane. The liquid was aspirated from the filter and the protein concentration measured using the Nanodrop 1 000 (Thermo Fisher Scientific) as described before. A stock solution of 1 mg/ml was prepared. One microlitre of 100 x protease inhibitor (Thermo Fisher Scientific) was added, and the protein was stored at -70°C until required.

2.2.3 Expression and purification of recombinant Marburg virus nucleoprotein

2.2.3.1 Cultivation and transfection of cells with Marburg virus nucleoprotein expression plasmids

Human embryonic kidney 293 cells with SV40 large T-antigen were prepared as described in section 2.2.2.1. Transfection of MARV Musoke NP expression plasmids (pCAGGS-MARV NP) or pCAGGS-MCS expression vector (control) into a 75 cm^2 cell culture flask of HEK 239T cells was performed when the cells reached approximately 80% confluency. The transfection agent was prepared by adding 19 µg purified plasmid DNA (1 mg/ml, from section 2.2.1.12) or pCAGGS-MCS plasmid DNA (1 mg/ml, negative control) and 57 µl *Trans*IT-LT1 transfection reagent (Mirus Bio LLC) to 1.9 ml 1 x Opti-MEM I containing HEPES, L-glutamine and 2.4 g/l sodium bicarbonate (Invitrogen). The mixture was incubated at room temperature for 30 minutes and added to the HEK 293T cells. The cells were incubated (Forma Series II, Thermo Fisher Scientific) at 37°C in a 5% CO_2 atmosphere for 72 hours.

2.2.3.2 Cesium chloride density gradient ultracentrifugation

After 72 hours of incubation at 37°C, the pCAGGS-MARV NP and pCAGGS-MCS transfected HEK 293T cells were lysed as follows: working on ice, 2.5 ml lysing agent (10 mM Tris-HCl (pH 7.8), 0.15 M NaCl, 1 mM EDTA, 0.1% Nonidet P-40 and protease inhibitor mixture (Roche)) was prepared in a 15 ml centrifuge tube (NEST). The supernatant was removed from the transfected HEK 293T cells, and 600 µl of the lysing agent was added to the cells. The cells were incubated for 30 minutes at 4°C. The cell lysate was collected into a 1.5 ml Eppendorf tube and mixed well. The tube was centrifuged (Sorvall Legend Micro 17, Thermo Fisher Scientific) at 13 800 x *g* for 15 minutes at 4°C. Cesium chloride (CsCl) (Sigma-Aldrich) solutions (15%, 25%, 35% and 40% w/v) were prepared in 50 mM Tris-buffered saline (TBS), pH 7.6 (Merck). A CsCl gradient was prepared in an ultracentrifuge tube (1/2 x 2UC tube, Beckmann Coulter) by carefully adding 1 ml of 40% CsCl, followed by 1 ml of 35% CsCl, 25% CsCl and finally 15% CsCl to the tube. The MARV NP or control cell lysate was carefully added on top of the CsCl gradient. The ultracentrifuge tube was placed in SW 55 Ti rotor adapters (Beckmann Coulter) and clipped onto the SW 55 Ti rotor. The tube was centrifuged at 327 327 x *g* for 2 hours at 4°C. Fractions of 500 µl each were collected from the tube by

carefully pipetting. Fractions were stored at 4°C until required for SDS-PAGE and western blotting.

2.2.3.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis, Coomassie staining and western blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed on the fractions collected in section 2.2.3.2 as described in section 2.2.2.4. Western blotting and Coomassie staining was performed as described in section 2.2.2.5. The expected band size for MARV NP was approximately 96 kDa.

2.2.3.4 Dialysis and ultrafiltration

Dialysis of the fractions containing NP was performed using the EasySep kit (TOMY) according to the manufacturer's instructions. Corresponding fractions of CsCl-purified control lysate was dialysed using the same procedure. Briefly, the correct fraction was applied to the dialysis membrane. The membrane was attached to a float and placed in 50 mM TBS, pH 7.6 (Merck) to wet the membrane. A tube was attached to the dialysis membrane, placed in the TBS on a float, and placed on a magnetic stirrer (Monostir, Rodwell Scientific Instruments Ltd) at 4°C for 6 hours. The TBS was replaced with a fresh batch and placed back onto the magnetic stirrer at 4°C overnight. The tube with the dialysis membrane was centrifuged (Sorvall Legend Micro 17, Thermo Fisher Scientific) at 13 800 x *g* for 1 minute. The protein concentration was measured using the Nanodrop 1 000 (Thermo Fisher Scientific) as described previously (section 2.2.2.6).

Ultrafiltration was performed using Amicon Ultra 0.5 ml centrifugal filters (Merck) according to the manufacturer's instructions. Briefly, the filter was inserted into a tube, and TBS (Merck) was applied to wet the membrane. The tube was centrifuged at 13 800 x *g* for 2 minutes. The TBS was discarded, and the sample containing the desired protein (from the dialysis step) was added to the tube containing the filter. The sample was centrifuged at 13 800 x *g* for 5 minutes. The filter was inverted and placed into a new tube. The tube was centrifuged at 13 800 x *g* for 5 minutes. The protein concentration was measured using the Nanodrop 1 000 (Thermo Fisher

Scientific) as described in section 2.2.2.6. One microlitre of 100 x protease inhibitor (Thermo Fisher Scientific) was added to the protein. The protein was stored at -70°C until required.

2.2.4 Optimisation and evaluation of indirect enzyme-linked immunosorbent assays

2.2.4.1 Test and control sera

Approval for using ERB sera in the development of I-ELISAs for MARV was obtained from the Department of Agriculture, Forestry and Fisheries of South Africa (12/11/1/1/13; Appendix A). Ethics approval for the use of bat serum in the evaluation of the I-ELISAs developed in this study was obtained from the University of Pretoria Animal Ethics Committee (EC056-14; Appendix A). Seven bat panels were used for the development and evaluation of the I-ELISAs. Briefly, a gamma-irradiated serum pool from ERBs inoculated with MARV during a previous experiment (Paweska *et al*., 2015) was used as a high positive control (panel 1). Negative control serum was obtained by pooling the sera of six captive-bred ERBs with no history of exposure to MARV (panel 1). Potential intra-colony transmission of MARV from bats collected in the wild to captive-bred ERBs was excluded by testing sera and swabs collected from these bats on a regular basis. For an assessment of the analytical specificity of each assay, serum specimens collected from bats seronegative to MARV and experimentally infected with EBOV/Hsap/GAB/96/Zaire-SPU220-96 (fourth passage in Vero cells) during a previous experiment (Paweska *et al*., 2016) were tested using both I-ELISAs (panel 2). Sera from wild-caught bats brought into captivity, and for which previous ELISA results were available, were used to assess the analytical sensitivities, robustness, repeatability and intermediate precision of the I-ELISAs (Appendix B) (panel 7). A large serum panel from wild-caught (Paweska *et al*., 2018) and experimentally MARV-infected bats (Paweska *et al*., 2015) was also used to compare results between the two assays (Appendix B) (panel 6). These sera were previously tested using an I-ELISA based on commercially available recombinant MARV GP (Integrated BioTherapeutics). Sera from captive juvenile ERBs collected at 5 and 7 months of age were used to determine the cut-off value for each assay (panel 3). For estimates of the diagnostic sensitivity and specificity of the I-ELISAs, serum specimens collected previously from experimentally MARV-infected bats (Paweska *et al*., 2015) (panel 4), real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)-negative control bats (Paweska *et*

al., 2015) (panel 5) and 7 month old captive juvenile MARV-naive bats (panel 3) were used. Further details of the bat serum panels are provided in Appendix B.

2.2.4.2 Optimisation of indirect enzyme-linked immunosorbent assays

Optimal reagent concentrations for the I-ELISAs were determined by means of standard checkerboard titration procedures (Crowther, 2009). Flat-bottom Maxisorp 96-well immunoplates (Nunc) were coated with 50 ng/100 µl/well purified recombinant MARV GP or NP antigen and pCAGGS control antigen (sections 2.2.2.6 and 2.2.3.4) diluted in DPBS pH 7.2 (Table 2.2), and incubated at 4°C overnight.

Table 2.2: Dilutions of reagents for the recombinant Marburg virus glycoprotein- and nucleoprotein-based indirect enzyme-linked immunosorbent assays

Indirect ELISA antigen	Concentration of recombinant/ control antigen	Dilution of recombinant/control antigen	Dilution of bat serum	Dilution of anti-bat conjugate (1 mg/ml stock)
Marburg virus Musoke GP	1 mg/ml	1:2 000 (50 ng/100 µl)	1:100	1:2 000 (50 ng/100µl)
Marburg virus Musoke NP	0.62 mg/ml	1:1 200 (50 ng/100 µl)	1:100	1:2 000 (50 ng/100 µl)
pCAGGS control	1 mg/ml	1:2 000 (50/ng/100 µl)	1:100	1:2 000 (50 ng/100µl)

Plates were washed three times using 300 µl/well PBS-T washing buffer (Sigma-Aldrich). Plates were then blocked using 200 µl/well of 10% fat-free milk powder in DPBS and incubated in a humid chamber for 1 hour at 37ºC. Plates were washed three times using 300 µl/well PBS-T. Control and test sera were diluted 1:100 (Table 2.2) in 2% fat-free milk powder in DPBS, and 100 µl/well was added to the plates. Test sera (section 2.2.4.1), conjugate controls (2% fat-free milk powder in DPBS) and negative control serum (panel 1; Appendix B) were assayed in duplicate, while positive control serum (panel 1; Appendix B) was assayed in quadruplicate. The plates were incubated at 37°C in a humid chamber for 1 hour. Plates were washed six times using 300 µl/well PBS-T. A volume of 100 µl/well goat anti-bat (1 mg/ml; Bethyl)

immunoglobulin G-horseradish peroxidase conjugate, diluted 1:2 000 in 2% fat-free milk powder in DPBS, was added to the plates. According to the manufacturer, this antibody conjugate specifically detects IgG of a variety of bat species including *Pteropus vampyrus*, *Desmodus rotundus*, *Eptesicus fuscus*, *Tadarida pumila*, *Hypsignathus monstrosus*, *Rousettus aegyptiacus*, *Epomophorus crypturus*, *Molossus molossus* and *Phyllostomus hastatus*. The plates were incubated at 37°C in a humid chamber for 1 hour. Plates were washed six times using 300 µl/well PBS-T. A volume of 100 µl/well pre-warmed 2,2'-Azino di-ethyl-benzothiazoline-sulfonic acid substrate (ABTS) was added to the plates, and plates were incubated in the dark at 25°C for 30 minutes. The reactions were stopped by adding 100 µl/well of 1% sodium dodecyl sulphate solution to the plates. Optical density (OD) values were measured at 405 nm using a microplate reader (ELx800, Bio-Tek Instruments Inc.). The means of the OD values of the test sera replicates were calculated and converted to a percentage positivity (PP) relative to the positive control serum using the following equation (Paweska *et al*., 2005):

$$Percentage\ positivity\ = \frac{Mean\ net\ OD\ of\ test\ sera\ replicates}{Mean\ net\ OD\ of\ positive\ control\ serum\ replicates} x\ 100$$

Conversion of raw OD values to PP values results in a continuous scale for I-ELISAs and is recommended by the World Organisation for Animal Health (OIE) (Wright *et al*., 1993). The method offers an advantage in that it does not assume uniform background activity and enables inter-laboratory assay standardisation (Wright *et al*., 1993).

2.2.4.3 Internal control limits

Upper and lower limits for the internal controls (high positive bat serum - C++; negative bat serum – C-) were determined by testing four (C++) or two (C-) replicates of the control sera on 20 plates during routine runs of the assay. Upper control limits (UCL) were determined by calculating the mean OD value from the control replicates plus two standard deviations, and

lower control limits (LCL) were determined by calculating the mean OD value from the control replicates minus two standard deviations.

2.2.4.4 Analytical specificity

The analytical specificity of each I-ELISA (the ability of the assay to distinguish between the analyte of interest and other components in a test specimen) was determined by testing five serum specimens from ERBs that had previously been experimentally infected with EBOV but were MARV seronegative and qRT-PCR negative (Paweska *et al*., 2016; panel 2; Appendix B).

2.2.4.5 Antibody dose/response curves

Antibody dose/response curves were generated for each I-ELISA using high positive and negative bat control sera, as well as five serum samples from captive wild-caught bats that previously tested positive for anti-MARV antibodies (low (PP value range: 17- 30), medium (PP value range: 31-70) or high antibody levels (PP value ≥ 71)) using an I-ELISA based on a commercially available recombinant MARV GP (Paweska *et al*., 2018; panel 7; Appendix B). The sera were serially diluted (two-fold) from a 1:100 dilution to extinction of the assay's response in 2% skim milk in DPBS, and the I-ELISAs were performed as described in 2.2.4.2.

2.2.4.6 Robustness

The robustness of the MARV GP and NP I-ELISAs (the ability of the assay to remain unaffected by minor variations in assay procedures) was evaluated by performing the I-ELISAs using four replicates of three bat specimens (negative serum (PP value < 17), low positive serum (PP value 17 – 30) and high positive serum (PP value ≥ 71) based on a commercial recombinant MARV GP-based I-ELISA (Integrated BioTherapeutics); panel 7; Appendix B) under variable conditions including incubation temperature (room temperature (25°C) versus 37°C) and incubation time variations (incubation times increased or decreased by 5 minutes). The I-ELISAs were performed as described in 2.2.4.2. Means, standard deviations (SD) and coefficients of variation (CV) of the PP values were calculated for the replicates tested with each variable. Coefficients of variation were calculated using the following formula:

$$\%CV = \frac{SD}{Mean\ PP} x\ 100$$

2.2.4.7 Repeatability and intermediate precision

To assess the repeatability (intra-plate variation) and intermediate precision (inter-plate variation) of each I-ELISA, ten bat serum specimens with antibody activity within the MARV NP and GP I-ELISA operating range were selected (panel 7; Appendix B). Of these specimens, five were negative sera, and the remaining five were sera containing low (PP value 17 – 30), medium (PP value 31-70) or high (PP value ≥ 71) anti-MARV IgG antibody levels based on previous results obtained from a commercial recombinant MARV GP-based I-ELISA (Integrated BioTherapeutics). Four replicates of each serum sample were prepared by creating individual working dilutions (1:100 in 2% skim milk in DPBS), and testing each replicate on five Maxisorp 96-well (Nunc) ELISA plates on five separate occasions. The means, SD and CV of the PP values were calculated from the replicates within each plate and between each run to determine the repeatability and intermediate precision of the assays.

2.2.4.8 Selection of cut-off values

The cut-off for each I-ELISA was determined by calculating the mean plus 3 SD from PP values obtained from testing 42 serum specimens of 5 to 7 month old juvenile ERBs that had been brought into captivity at approximately 2 months of age, with no previous exposure to MARV (panel 3; Appendix B).

2.2.4.9 Diagnostic sensitivity and specificity

Estimates of the diagnostic sensitivity (the proportion of known positive sera that test positive using the assay) and specificity (the proportion of known negative sera that test negative using the assay) of each I-ELISA were determined by testing 81 sera from known MARV-positive ERBs (bats experimentally infected with MARV and that tested positive by qRT-PCR and/or virus isolation) (Paweska *et al*., 2015; this thesis chapter 3; panel 4; Appendix B) and 96 sera

from known negative ERBs (7 month old captive juvenile bats with no residual maternal antibodies to MARV, bats that served as controls during previous experimental MARV infection studies and that tested negative by qRT-PCR (Paweska *et al*., 2015; this thesis chapter 3; panel 3; Appendix B), and bats inoculated with EBOV in a previous study that tested negative for MARV by qRT-PCR (Paweska *et al*., 2016; panel 5; Appendix B)). Sera were collected from bats of different ages and sexes, and at different stages of infection. Diagnostic sensitivity and specificity estimates were determined using the following formulae (Jacobsen, 1998):

$$Diagnostic\ Sensitivity = \frac{True\ Positives}{(True\ Positives + False\ Negatives)} x\ 100$$

$$Diagnostic\ Specificity = \frac{True\ Negatives}{True\ Negatives + False\ Positives} x\ 100$$

2.2.4.10 Comparison of Marburg virus glycoprotein- and nucleoprotein-based indirect enzyme-linked immunosorbent assay performance in naturally and experimentally infected Egyptian rousette bats

A panel of 652 bat sera, including 480 serum specimens from wild-caught ERBs (287 females and 193 males; 40 adults, 82 juveniles and 232 sub-adults; panel 6; Appendix B), 146 serum specimens from experimentally MARV-infected ERBs (10 females and 9 males; 4 juveniles and 15 adults; panel 4; Appendix B) or control bats (7 males and 6 females, all adult; panel 5; Appendix B), and 26 serum specimens from juvenile ERBs brought into captivity at approximately 2 months of age (13 males and 13 females; panel 3; Appendix B) and that had lost their maternal immunity, was tested to compare results between the two I-ELISAs. The results were further compared to results obtained using an I-ELISA based on commercially available recombinant MARV GP (Integrated BioTherapeutics).

2.2.4.11 Predictive values of positive and negative test results

The apparent prevalence of MARV exposure was determined using the data obtained from testing the sera of 480 wild-caught ERBs with each I-ELISA by dividing the number of test positives with the number of bats tested. Subsequently, the following calculations were performed (Jacobsen, 1998):

$$Number\ of\ bats\ exposed = Number\ of\ bats\ tested\ x\ apparent\ prevalence$$
$$Number\ of\ true\ positive\ tests = Diagnostic\ sensitivity\ x\ number\ of\ bats\ exposed$$
$$Number\ of\ false\ negative\ tests = Number\ of\ bats\ exposed - number\ of\ true\ positive\ tests$$
$$Number\ of\ bats\ unexposed = Number\ of\ bats\ tested - number\ of\ bats\ exposed$$
$$Number\ of\ true\ negative\ tests = Diagnostic\ specificity\ x\ number\ of\ bats\ unexposed$$
$$Number\ of\ false\ positive\ tests = Number\ of\ bats\ unexposed - number\ of\ true\ negative\ tests$$

Finally, the positive predictive values (PPV; the probability that a subject which tests positive using the assay truly has been exposed to the virus) and negative predictive values (NPV; the probability that a subject which tests negative using the assay truly has not been exposed to the virus) of the recombinant MARV GP- and NP-based I-ELISAs were determined using the following formulae (Jacobsen, 1998):

$$Positive\ predictive\ value = \frac{Number\ of\ true\ positive\ tests}{Number\ of\ true\ positive\ tests + Number\ of\ false\ positive\ tests} x\ 100$$

$$Negative\ predictive\ value = \frac{Number\ of\ true\ negative\ tests}{Number\ of\ true\ negative\ tests + Number\ of\ false\ negative\ tests} x\ 100$$

2.2.4.12 Antibody dynamics to the Marburg virus nucleoprotein and glycoprotein in experimentally infected Egyptian rousette bats

In order to identify the antibody dynamics of experimentally infected bats against the MARV GP and NP proteins, 75 serum specimens collected from 14 experimentally MARV-infected

ERBs (8 males, 6 females, 4 bats less than 1 year old, 10 bats more than 1 year old) (panel 4; Appendix B) over the course of 42 days were tested. The serum was obtained from a previous study in which captive-bred MARV-naive bats were subcutaneously inoculated with MARV (isolate SPU 148/99/I Watsa) (Paweska *et al*., 2015) at the CEZPD, NICD-NHLS. Indirect ELISAs were performed as described in 2.2.4.2.

2.2.4.13 Statistical analysis

Statistical analysis and basic calculations of means, SDs and CVs were performed in Microsoft Excel. Estimates of the diagnostic sensitivity and specificity of each assay were determined at the 95% confidence interval. Agreement between test results obtained from the commercial recombinant MARV GP-based I-ELISA (Integrated BioTherapeutics), and the MARV GP and NP I-ELISAs developed in this study was determined using Lin's Concordance Correlation Coefficient (CCC) (MedCalc version 18.2.1; www.medcalc.org). The Student's t-test was performed to determine whether there was a statistically significant difference in the mean levels of anti-MARV NP and anti-MARV GP antibodies in the experimentally infected bats over the course of 42 days (two-tailed p-value < 0.05).

2.3 Results

2.3.1 Amplification, cloning and sequencing of Marburg virus glycoprotein and nucleoprotein genes

The MARV Musoke GP and NP genes were successfully amplified using the primer sets described in Table 2.1, with agarose gel electrophoresis revealing bands of approximately 1 980 bp (GP) and 2 100 bp (NP) alongside a positive control band of 800 bp produced using M13 forward and reverse primers (results not shown). To aid in sequencing and efficient restriction enzyme digestion, the inserts were initially cloned into the pCR-Blunt II-TOPO cloning vector. Following transformation of *E. coli* and overnight incubation on LB agar, no colonies formed on the negative control plate (untransformed *E. coli*) or on the plate with *E. coli* transformed with vector only, while more than 50 colonies formed on the plates with transformed *E. coli* containing the pCR-Blunt II-TOPO vector plus either the positive control, MARV GP or MARV NP insert. The transformation control (pUC19 vector) plate contained

more than 100 colonies. Polymerase chain reaction analysis using M13 primers revealed colonies containing TOPO plasmids with the desired inserts (MARV GP - 2 223 bp; MARV NP - 2 360 bp; positive control - 800 bp). Subsequent sequencing using M13 primers further revealed colonies with inserts cloned into the TOPO vector in the correct orientation. The nucleotide sequences of the cloned genes were evaluated against that of the MARV Musoke reference strain available in the public domain (GenBank, www.ncbi.nlm.noh.gov; accession number NC_001608.3) (Appendix C and D). Plasmids were successfully isolated from colonies in which no difference in the sequences could be observed between the MARV GP and NP genes and the MARV Musoke reference strain. Agarose gel electrophoresis of the plasmids revealed bands of the correct sizes (5 500 bp for TOPO-MARV GP and 5 600 bp for TOPO-MARV NP) (results not shown).

Following isolation of the TOPO-MARV GP and TOPO-MARV NP plasmids, the MARV GP and NP genes were sub-cloned into the pCAGGS-MCS expression vector using restriction enzyme digestion and ligation. Digestion of the TOPO-MARV GP plasmid using EcoRI and NheI restriction enzymes resulted in two bands of approximately 3 500 bp (pCR-Blunt II-TOPO vector backbone) and 2 000 bp (MARV GP gene), and digestion of the TOPO-MARV NP plasmid resulted in two bands of approximately 3 500 bp (pCR-Blunt II-TOPO vector backbone) and 2 100 bp (MARV NP gene) following agarose gel electrophoresis. Restriction enzyme digestion of the pCAGGS-MCS expression vector using the same enzymes revealed a band of approximately 4 700 bp (digested pCAGGS-MCS backbone) upon agarose gel electrophoresis analysis. Purified MARV GP and NP inserts were ligated into the digested pCAGGS-MCS expression vector, and DH5α *E. coli* were transformed using the resulting plasmids. After overnight incubation on LB agar containing Ampicillin, several colonies (~20) were observed on each plate containing the pCAGGS-MARV GP, pCAGGS-MARV NP and pCAGGS-MCS transformants, and none were observed on the control plates with untransformed *E. coli* or *E. coli* transformed with digested pCAGGS vector only. Polymerase chain reaction performed using pCAGGS-specific primers, and agarose gel electrophoresis analysis of colonies revealed that sub-cloning of the inserts into the pCAGGS expression vector was successful (Figure 2.3; expected band sizes were approximately 2 123 bp for MARV GP and 2 259 bp for MARV NP). Subsequent sequencing using pCAGGS-specific primers indicated that the inserts were present in the correct orientation in the expression plasmid, with

no differences in the sequences of the MARV GP and NP genes and the MARV Musoke reference strain.

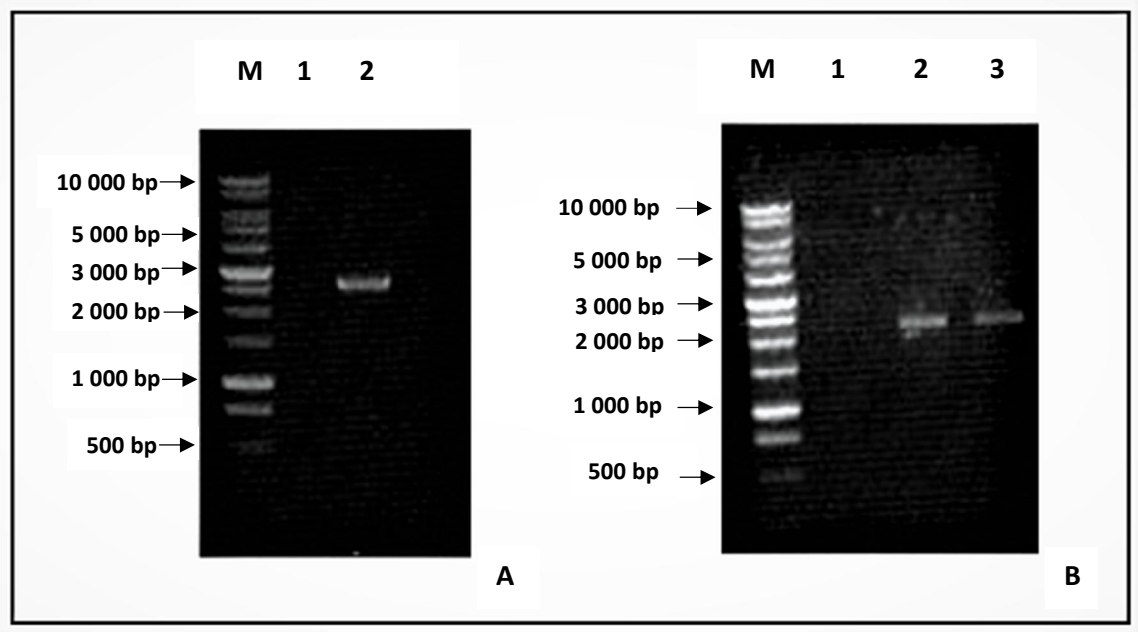


Figure 2.3: Amplification of the Marburg virus (MARV) Musoke genes from *Escherichia coli* successfully transformed with pCAGGS-MARV glycoprotein (GP) and pCAGGS MARV nucleoprotein (NP) plasmids using pCAGGS-specific primers. The presence of bands indicates the presence of the gene of interest. A) Nucleoprotein. M = 1 kb marker (Nippon Genetics), 1 = colony without insert, 2 = colony with the correct size insert (MARV NP - 2 259 bp). B) Glycoprotein. M = 1 kb marker (Nippon Genetics), 1 = colony without insert, 2, 3 = colonies with the correct size insert (MARV GP – 2 123 bp).

The pCAGGS-MARV GP and pCAGGS-MARV NP expression plasmids were successfully isolated from the correct colonies using a maxiprep plasmid DNA purification kit. Agarose gel electrophoresis of the plasmids revealed the expected band sizes of approximately 6 700 bp (pCAGGS-MARV GP), 6 800 bp (pCAGGS-MARV NP) and 4 700 bp (pCAGGS-MCS).

2.3.2 Expression and purification of recombinant Marburg virus Musoke proteins

Human embryonic kidney 293T cells were transfected with the pCAGGS-MARV GP expression plasmid or pCAGGS-MCS control plasmid using linear polyethylenimine as a transfection reagent. After 48 hours of incubation, the presence of expressed MARV GP in the supernatant of the pCAGGS-MARV transfected cells, but not in the pCAGGS-MCS transfected cells, was confirmed by western blot (insufficient resolution of the Coomassie-stained SDS-PAGE gel warranted use of the more sensitive western blot technique) (results not shown). The His-tagged MARV GP was subsequently purified from the supernatant using Ni-NTA agarose resin. Western blot analysis revealed the presence of purified MARV GP (~150 kDa (GP_1) and ~40 kDa (GP_2 without 5 kDa transmembrane domain and with 1 kDa His-tag), as expected) in the first elution fraction following binding to the Ni-NTA resin and wash steps, but not in the corresponding fraction of supernatant from cells transfected with pCAGGS-MCS purified in the same manner (Figure 2.4). The heavily glycosylated nature of MARV GP resulted in slowed migration of the protein in the SDS-PAGE gel, resulting in the diffuse appearance of the bands on the blot (Martina *et al*., 1998; Gerpe *et al.*, 2015). Following dialysis and ultrafiltration, approximately 1 mg/ml of MARV GP could be purified from the pCAGGS-MARV GP-transfected cell supernatant.

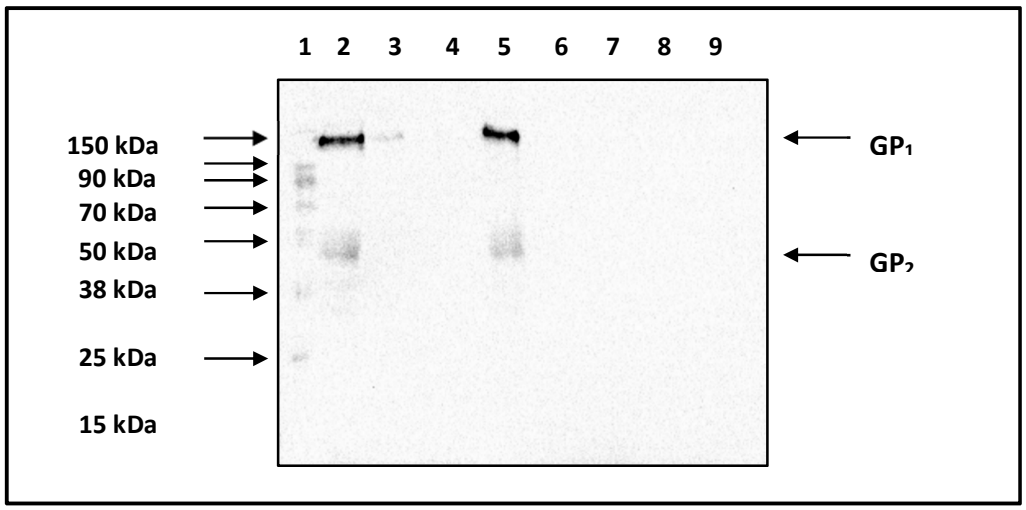


Figure 2.4: Western blot showing the successful purification of Histidine-tagged Marburg virus glycoprotein (GP_1 ~ 150 kDa; GP_2 ~ 40 kDa) using nickel nitrilotriacetic acid agarose resin. Lane 1 = chemiluminescent protein marker (LiCor); lane 2 = Marburg virus (MARV) glycoprotein (GP) supernatant pre-purification; lane 3 = eluate post binding to column; lane 4 = wash step eluate; lane 5 = MARV GP elution; lane 6 = pCAGGS control supernatant pre-purification; lane 7 = control eluate post binding to column; lane 8 = control wash step eluate; lane 9 = pCAGGS control elution.

Trans-IT LT1 transfection reagent was used to transfect HEK 293T cells with the pCAGGS-MARV NP expression plasmid or pCAGGS-MCS control plasmid. After 72 hours of incubation, expressed MARV NP was detected in the lysate of pCAGGS-MARV NP transfected cells by Coomassie staining of an SDS-PAGE gel and western blot (results not shown). The MARV NP was subsequently purified from the cell lysate using CsCl density gradient ultracentrifugation. Purified MARV NP (~100 kDa) could be detected in fractions five to seven, and no protein was detected in the corresponding fractions of pCAGGS-MCS-transfected cell lysate purified in the same manner (Figure 2.5). Pooling of the fractions in which MARV NP was present, followed by dialysis and ultrafiltration, yielded approximately 600 µg/ml protein.

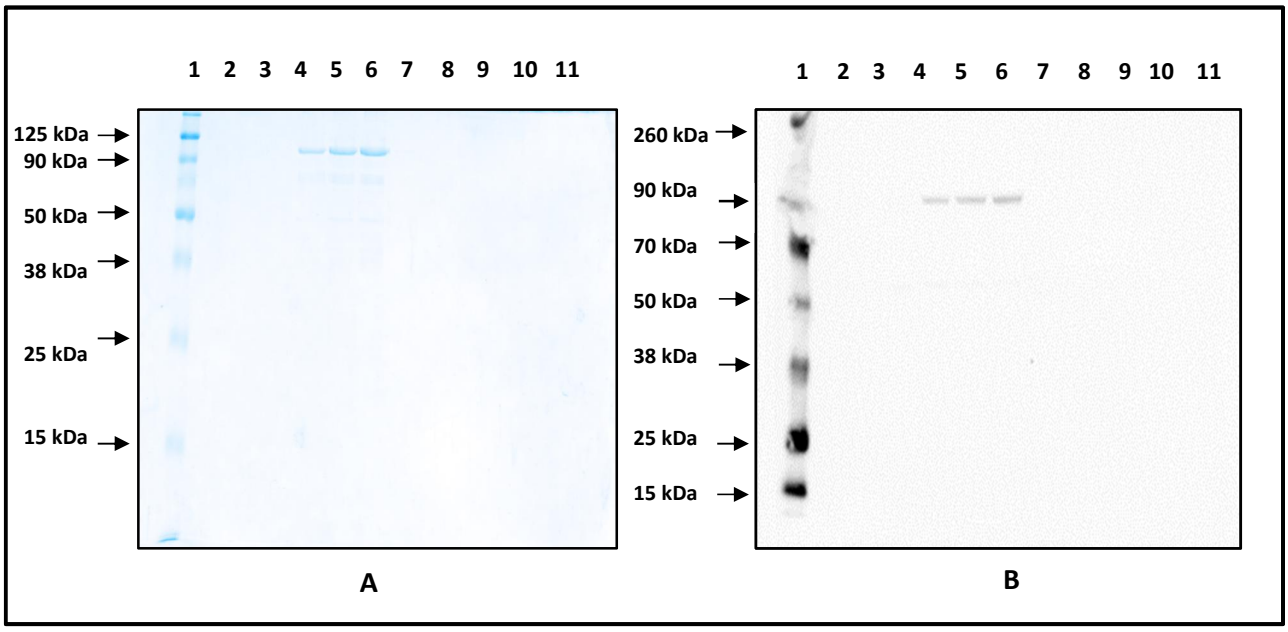


Figure 2.5: Coomassie stained SDS-PAGE gel (A) and western blot (B) showing the successful purification of Marburg virus (MARV) nucleoprotein (NP) (~100 kDa) using cesium chloride density gradient ultracentrifugation. Lane 1 = chemiluminescent protein marker (LiCor); lane 2-6 = NP purification fractions 3-7; lane 7-11 = pCAGGS purification fractions 3-7.

The MARV GP, NP and pCAGGS controls were sufficiently expressed and purified by the respective methods for use as antigens or control antigens in subsequent serological assays.

2.3.3 Evaluation of indirect enzyme-linked immunosorbent assays

2.3.3.1 Internal control limits

Both the MARV GP- and NP-based I-ELISAs could clearly differentiate between high positive and negative controls, with little variation in the OD values of the internal controls within and between runs (Figure 2.6 and 2.7). Upper control limits for the high positive control were established at OD values of 1.3 and 1.7 for the MARV GP- and NP-based assays, respectively, while LCL were established at OD values of 1.1 and 1.5 for the MARV GP- and NP-based I-ELISAs, respectively.

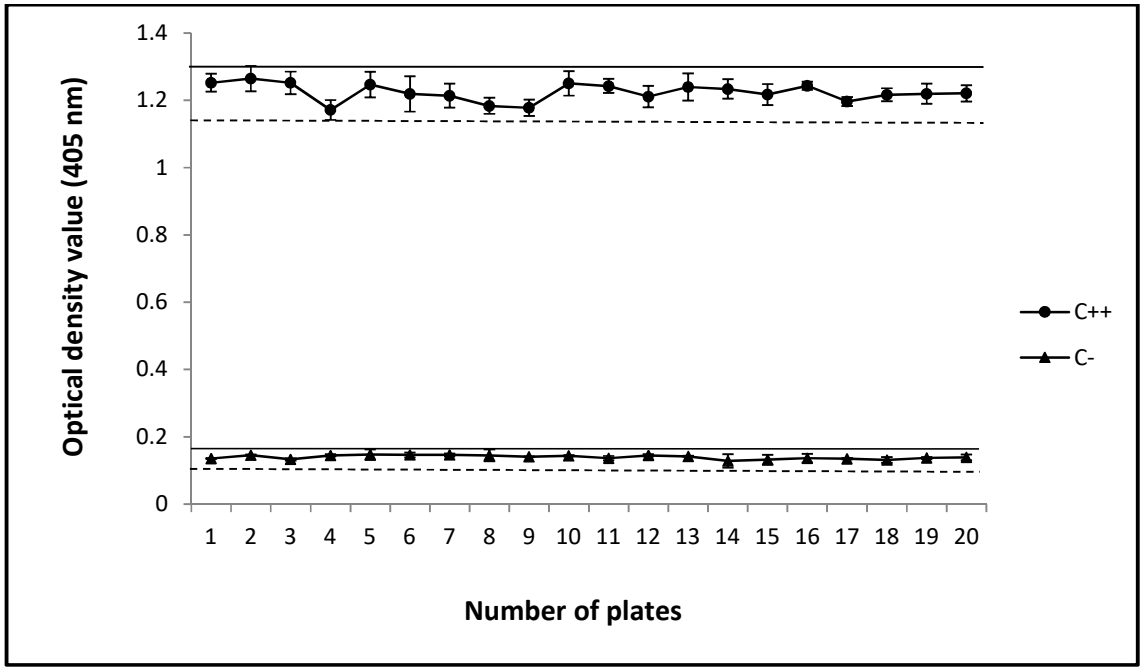


Figure 2.6: Upper (—) and lower (- - -) internal control limits for optical density values of high positive (C++) and negative serum (C-) in the recombinant Marburg virus glycoprotein-based indirect enzyme-linked immunosorbent assay. Results are shown for the controls (mean ± standard deviation (represented as error bars)) tested in 20 plates during routine runs of the assay.

The upper control limit for the negative control was established at an OD value of 0.2 for both the MARV GP- and NP-based I-ELISAs, while the LCL was established at an OD value of 0.1.

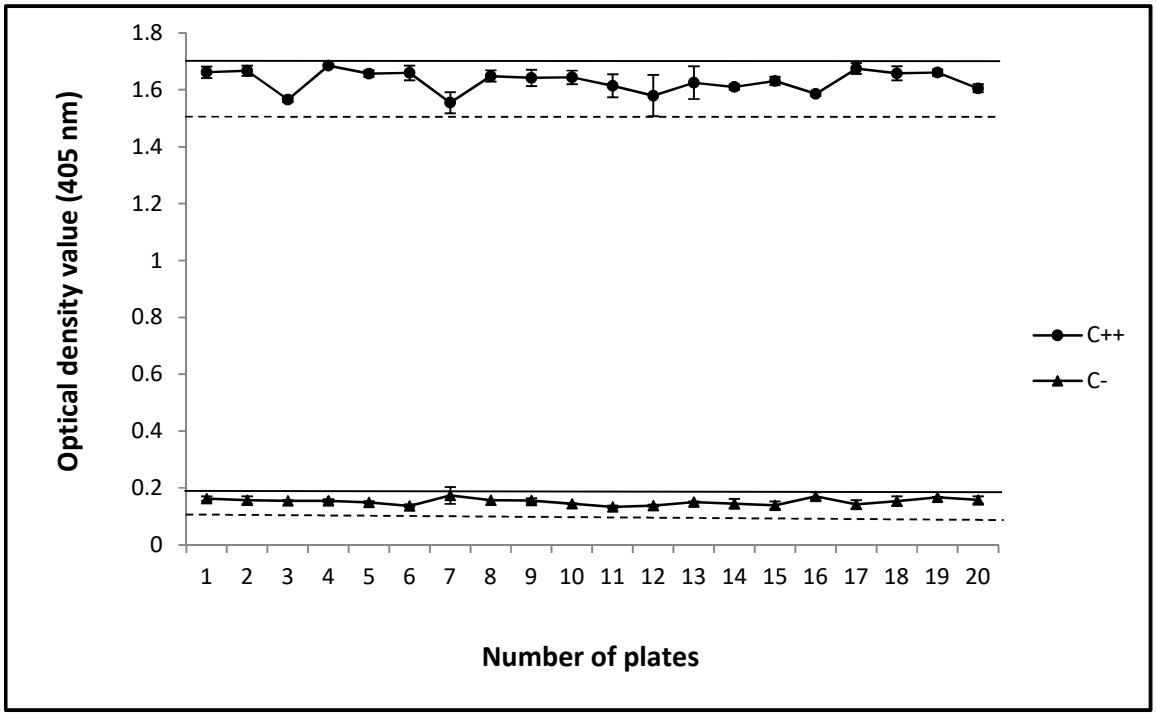


Figure 2.7: Upper (—) and lower (- - -) internal control limits for optical density values of high positive (C++) and negative serum (C-) in the recombinant Marburg virus nucleoprotein-based indirect enzyme-linked immunosorbent assay. Results are shown for the controls (mean ± standard deviation (represented as error bars)) tested in 20 plates during routine runs of the assay.

2.3.3.2 Analytical specificity

There was no cross-reaction between the EBOV positive bat sera and the recombinant MARV GP antigen; however, cross-reaction occurred between 3/5 (60%) EBOV positive bat sera and the recombinant MARV NP antigen (mean PP value: 29.64) (Figure 2.8).

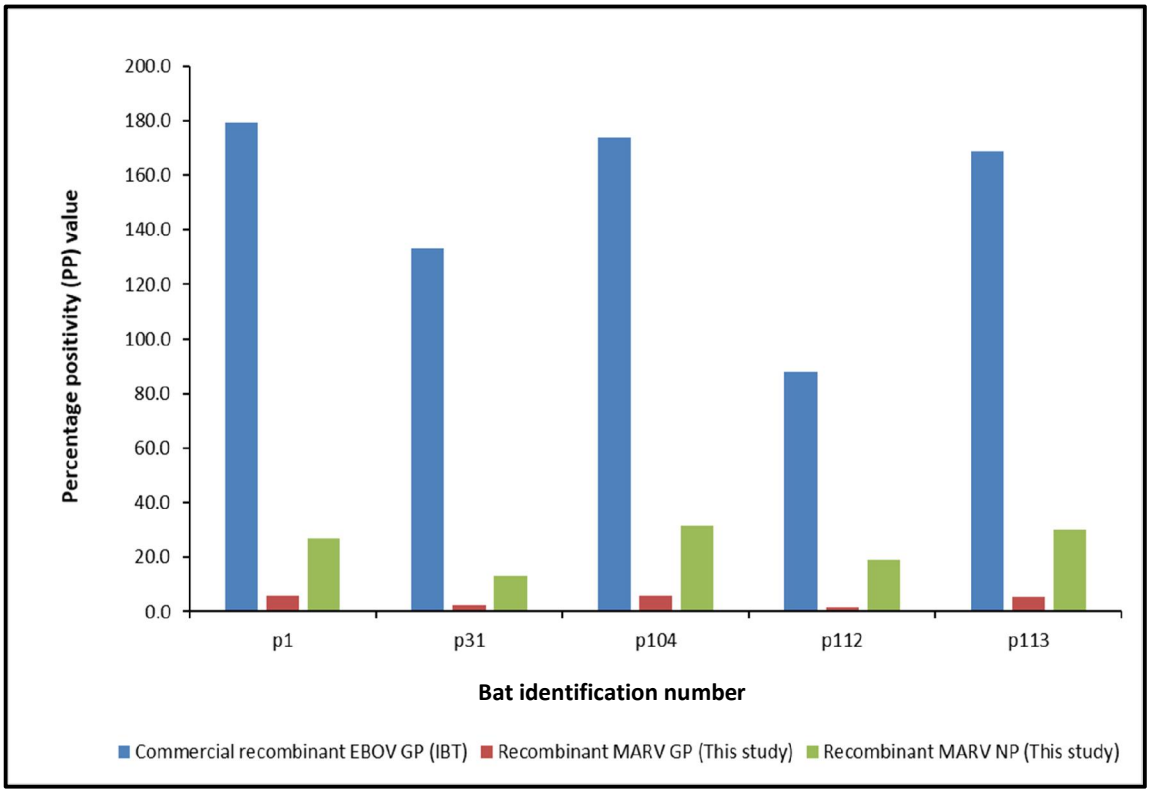


Figure 2.8: Serological reactivity of sera from Egyptian rousette bats experimentally infected with Ebola virus (EBOV) to commercially available recombinant EBOV glycoprotein (GP) and the recombinant Marburg virus (MARV) GP and nucleoprotein (NP) developed in this study.

2.3.3.3 Antibody dose/response curves

Dose/response curves produced from different serum dilutions from bats known to be either positive or negative for anti-MARV antibodies had the expected slope. Both the MARV GP and NP I-ELISAs could clearly differentiate between different levels of MARV-specific IgG antibody in ERBs with minimal background (Figure 2.9 and 2.10).

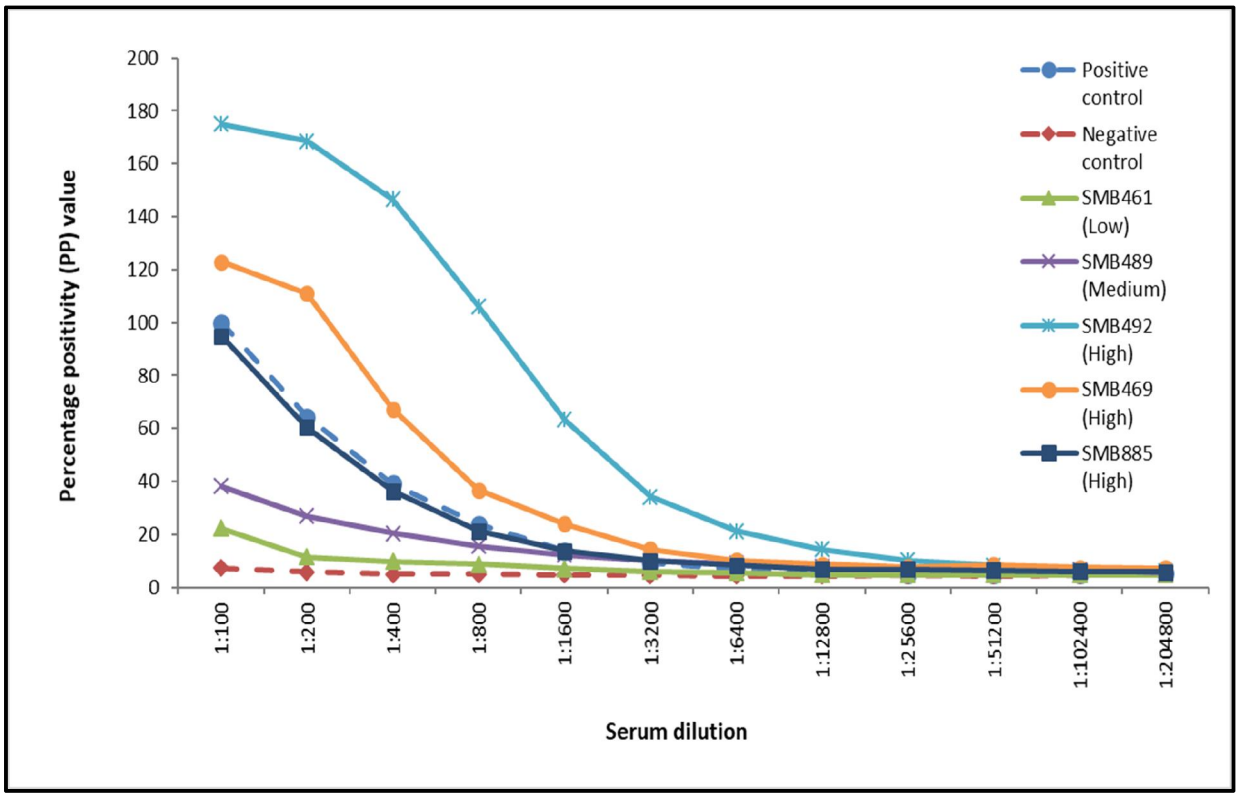


Figure 2.9: Dose response curves of individual Egyptian rousette bat sera in the recombinant Marburg virus (MARV) glycoprotein indirect enzyme-linked immunosorbent assay. The sera tested included positive and negative control bat serum, as well as sera from an additional five bats known to have varying levels (low, medium, high) of antibody to MARV.

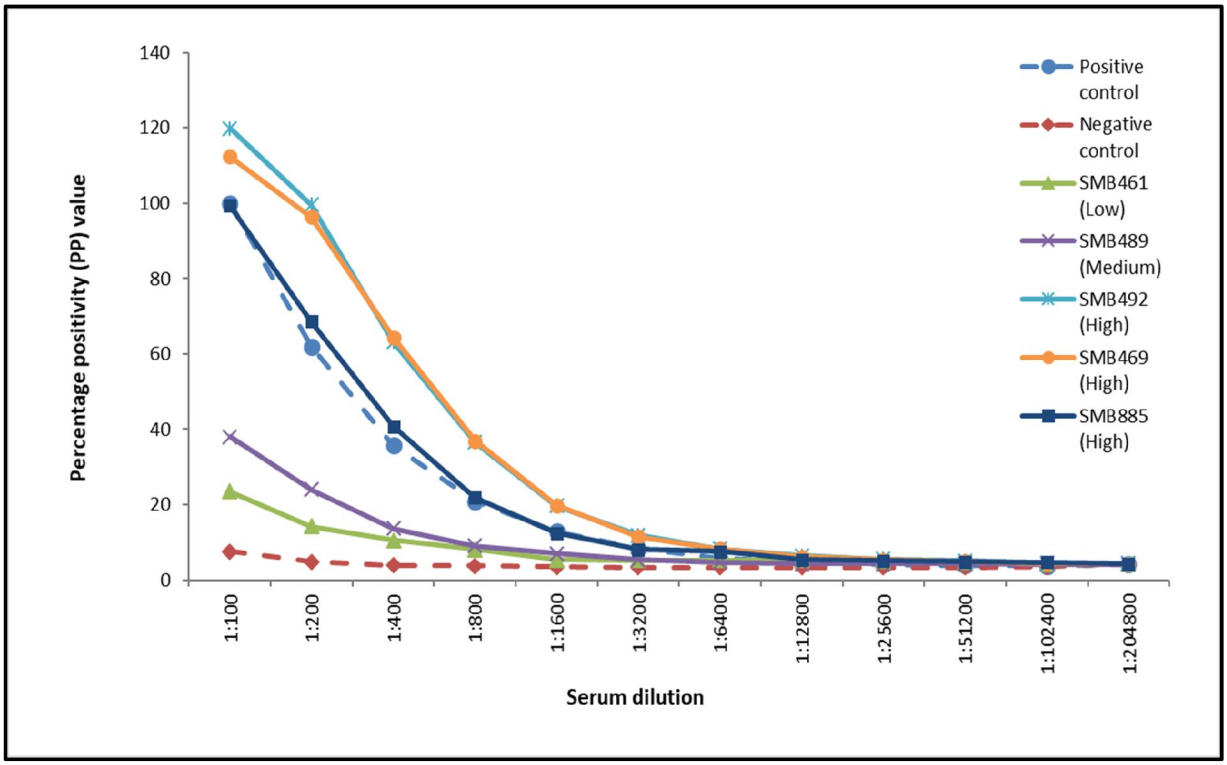


Figure 2.10: Dose response curves of individual Egyptian rousette bat sera in the recombinant Marburg virus (MARV) nucleoprotein-based indirect enzyme-linked immunosorbent assay. The sera tested included positive and negative control bat serum, as well as sera from an additional five bats known to have varying levels (low, medium, high) of antibody to MARV.

2.3.3.4 Robustness

Variation in incubation time and temperatures did not significantly influence the measured OD values of the three bat serum specimens obtained with the MARV GP or NP I-ELISAs (Table 2.3). For each variable applied to each I-ELISA, the CV was within acceptable limits (below 15%).

Table 2.3: Robustness coefficients of variation of serum specimens with varying anti-Marburg virus antibody concentrations

		Serum anti-Marburg virus antibody concentration		
Marburg virus glycoprotein ELISA		**High**	**Low**	**Negative**
Optimised protocol[a] versus longer incubation time	Mean[b]	159.6	20	9.9
	SD	2.7	1	0.2
	%CV	1.7	5	2
Optimised protocol versus shorter incubation time	Mean	156.9	18.2	9.7
	SD	1.4	1.3	0.2
	%CV	0.9	7.1	2.1
Optimised protocol versus room temperature incubation	Mean	152.7	18.3	9.6
	SD	5.6	1.2	0.2
	%CV	3.7	6.6	2.1

Marburg virus nucleoprotein ELISA		High	Low	Negative
Optimised protocol versus longer incubation time	Mean	171.2	26.2	10.5
	SD	8.7	1.2	0.3
	%CV	5.1	4.6	2.9
Optimised protocol versus shorter incubation time	Mean	162.2	24.6	10.2
	SD	1	2.1	0.2
	%CV	0.6	8.5	2
Optimised protocol versus room temperature incubation	Mean	160.2	22.3	10.1
	SD	3.8	2.9	0.3
	%CV	2.4	13	3

[a]Optimised protocol refers to the protocol as described in 2.2.4.2 of this thesis

[b]The mean percentage positivity (PP) value and SD were calculated from four replicates of each serum specimen per run

Abbreviations: ELISA – enzyme-linked immunosorbent assay, SD – standard deviation, %CV – percentage coefficient of variation

2.3.3.5 Repeatability and intermediate precision

Serum specimens with high, medium or low concentrations of anti-MARV antibody, as well as negative serum specimens, were used to assess the repeatability and intermediate precision of the MARV GP and NP I-ELISAs. The CV was acceptable for both I-ELISAs, with inter-plate and intra-plate CVs being below 10% (Table 2.4).

Table 2.4: Repeatability and intermediate precision coefficients of variation of serum specimens with varying anti-Marburg virus antibody concentrations

	Serum anti-Marburg virus antibody concentration	Repeatability			Intermediate precision		
		Mean PP*	SD	%CV	Mean PP	SD	%CV
Marburg virus glycoprotein ELISA	Medium	33.5	3.3	9.8	31.8	2.1	6.6
	High	74.1	2.3	3.1	75.2	2	2.7
	Negative	12.4	0.4	3.2	12.4	0.7	5.7
	Low	18.5	0.4	2.2	17.4	0.8	4.6
	Negative	9.2	0.2	2.2	9.2	0.6	6.5
	High	185.9	5.4	2.9	186.5	5.4	2.9
	Negative	13.3	0.5	3.8	11.5	1	8.7
	Negative	13.4	0.8	6	14.7	1.2	8.2
	Negative	11.5	0.2	1.7	10.4	0.8	7.7
	Medium	31.2	2.1	6.7	31.9	2	6.3

	Serum anti-Marburg virus antibody concentration	Repeatability			Intermediate precision		
		Mean PP*	SD	%CV	Mean PP	SD	%CV
Marburg virus nucleoprotein ELISA	Medium	42.8	2.5	5.8	41.5	2.9	7
	High	165.5	2.4	1.5	162.1	4.8	3
	Negative	12.6	0.4	3.2	12.5	0.3	2.4
	Low	28.4	0.9	3.2	27.3	1.4	5.1
	Negative	7.8	0.2	2.6	7.9	0.4	5.1
	High	170.3	3.2	1.9	160	6.4	4
	Negative	12	0.8	6.7	11.4	1.1	9.7
	Negative	13.3	0.7	5.3	12.9	0.8	6.2
	Negative	16.4	0.9	5.5	16.5	1.6	9.7
	Medium	36.3	2.4	6.6	35.9	2.4	6.7

Abbreviations: ELISA – enzyme-linked immunosorbent assay, PP – Percentage positivity, SD – standard deviation, %CV – percentage coefficient of variation

*The mean PP value and SD were calculated from four replicates of each serum specimen within each run (repeatability) and between runs (intermediate precision)

2.3.3.6 Indirect enzyme-linked immunosorbent assay cut-off values

The cut-off for each I-ELISA was determined by calculating the mean PP value plus 3 SD from 42 sera collected from 5 to 7 month old juvenile ERBs brought into captivity. The cut-off value

for the indirect recombinant MARV NP I-ELISA was determined to be 25.7, while the cut-off value for the indirect recombinant MARV GP I-ELISA was 17.1 (Figure 2.11).

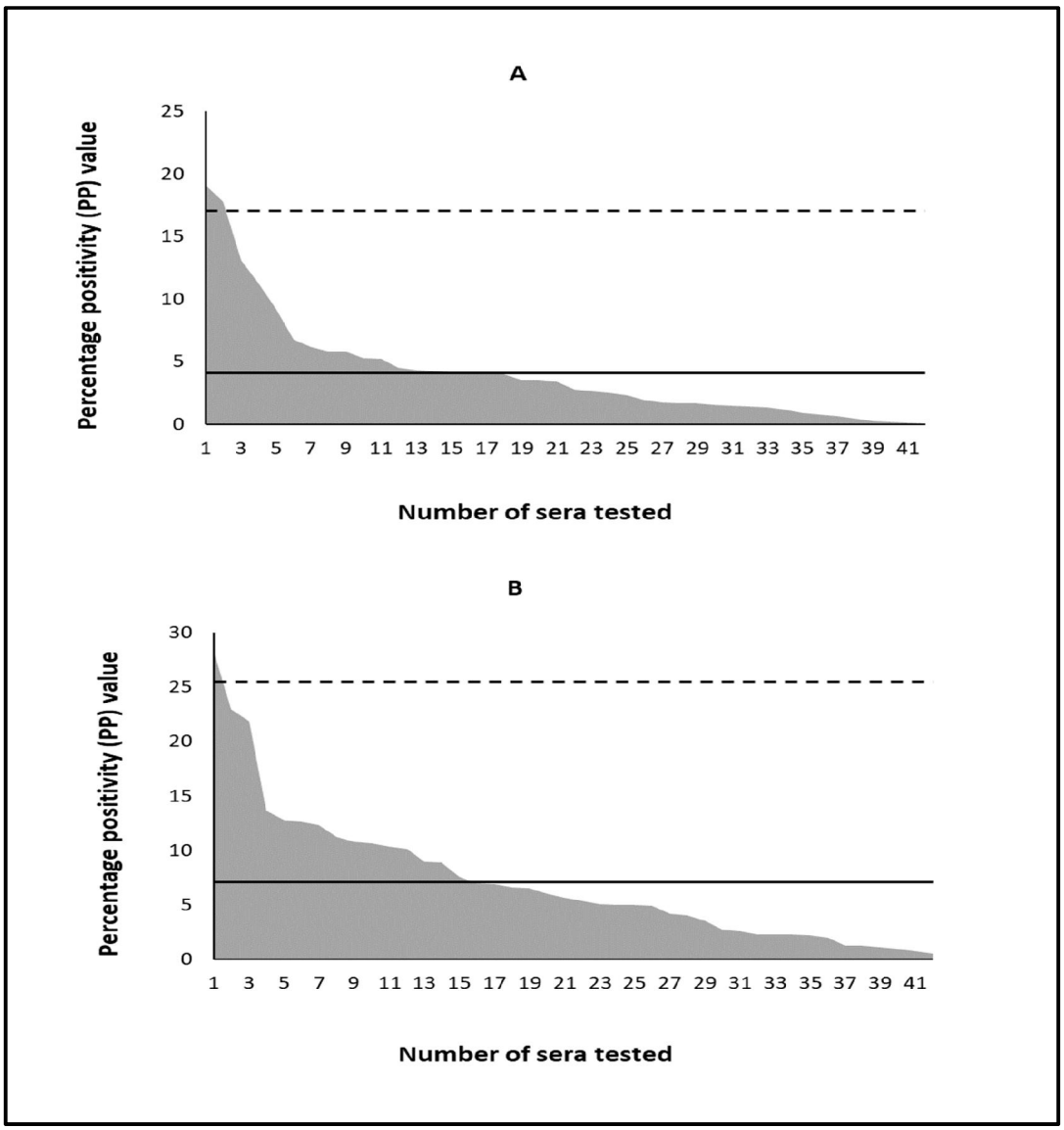


Figure 2.11: Distribution of percentage positivity (PP) values of sera from juvenile Egyptian rousette bats tested using the recombinant Marburg virus glycoprotein- (A) and nucleoprotein-based (B) indirect enzyme-linked immunosorbent assay, ordered from largest to smallest. The mean PP value for each assay is indicated with a solid black line, while the cut-off value for each assay, determined from the mean PP value plus three standard deviations, is indicated with a dashed black line.

2.3.3.7 Diagnostic sensitivity and specificity

Table 2.5 and 2.6 indicate the estimates of diagnostic sensitivity and specificity of the MARV GP I-ELISA and MARV NP I-ELISA, respectively. For the MARV GP I-ELISA, the diagnostic sensitivity was determined to be 98.8%, while diagnostic specificity was 100%.

Table 2.5: Diagnostic sensitivity and specificity estimates of the Marburg virus glycoprotein-based indirect enzyme-linked immunosorbent assay calculated from results for sera tested from known infected and uninfected Egyptian rousette bats

		Known positive (n=81)		Known negative (n=96)	
Test results	Positive	80	TP	FP	0
	Negative	1	FN	TN	96
		Diagnostic sensitivity* = (TP/(TP+FN) x 100 = 98.8%		Diagnostic specificity* = (TN/(TN+FP) x 100 = 100%	

Abbreviations: n – number; TP – true positive; FP – false positive; FN – false negative; TN – true negative

*Based on the following parameters:

1) Diagnostic sensitivity and specificity estimated at 95% prior to testing

2) Confidence of 95% required in diagnostic sensitivity and specificity estimates

3) Error margin of 5% allowed in estimating diagnostic sensitivity and specificity estimates

The diagnostic sensitivity and specificity of the MARV NP I-ELISA was lower, with estimates calculated at 96.3% and 96.9%, respectively (Table 2.6).

Table 2.6: Diagnostic sensitivity and specificity estimates of the Marburg virus nucleoprotein-based indirect enzyme-linked immunosorbent assay calculated from results for sera tested from known infected and uninfected Egyptian rousette bats

		Known positive (n=81)		Known negative (n=96)	
Test results	Positive	78	TP	FP	3
	Negative	3	FN	TN	93
		Diagnostic sensitivity* = (TP/(TP+FN) x 100 = 96.3%		Diagnostic specificity* = (TN/(TN+FP) x 100 = 96.9 %	

Abbreviations: n – number; TP – true positive; FP – false positive; FN – false negative; TN – true negative

*Based on the following parameters:

1) Diagnostic sensitivity and specificity estimated at 95% prior to testing

2) Confidence of 95% required in diagnostic sensitivity and specificity estimates

3) Error margin of 5% allowed in estimating diagnostic sensitivity and specificity estimates

2.3.3.8 Comparison of Marburg virus glycoprotein- and nucleoprotein-based indirect enzyme-linked immunosorbent assay performance in naturally and experimentally infected Egyptian rousette bats

To assess and compare the diagnostic and field performance of the I-ELISAs developed in this study, 652 specimens collected from bats experimentally infected with MARV, wild-caught bats, juvenile bats that have lost their maternal immunity and bats that served as controls during experimental inoculation studies, were tested. A summary of the results obtained from testing the different serum panels is given in Table 2.7. The results obtained from the I-ELISAs developed in this study were compared to results obtained using an I-ELISA based on a commercially available MARV GP recombinant antigen (Integrated BioTherapeutics). Results of individual sera from each serum panel are shown in Appendix B.

Table 2.7: Results obtained from testing sera of experimentally Marburg virus (MARV)-infected (n = 81) and control Egyptian rousette bats (ERBs) (n = 65), 7 month old captive juvenile ERBs (n = 26) and field-sampled ERBs (n = 480) using the recombinant MARV glycoprotein and nucleoprotein-based indirect enzyme-linked immunosorbent assays developed in this study

	Previous studies (Paweska *et al.*, 2015; Paweska *et al.*, 2018)	**This study**	
Bat group	**Commercial MARV GP I-ELISA result** (number of sera that tested positive/total number of sera tested)	**MARV GP I-ELISA result** (number of sera that tested positive/total number of sera tested)	**MARV NP I-ELISA result** (number of sera that tested positive/total number of sera tested)
Unexposed juvenile bats (qRT-PCR negative) (panel 3)	0/26	0/26	0/26
Experimentally infected (VI and/or qRT-PCR positive) (panel 4)	81/81	80/81	78/81
Control bats (qRT-PCR negative) (panel 5)	0/65	0/65	0/65
Field-sampled bats (panel 6)	192/480	193/480	187/480

Abbreviations: MARV - Marburg virus, GP - Glycoprotein, NP - Nucleoprotein, VI - Virus isolation, qRT-PCR – Real-time quantitative polymerase chain reaction

Lin's concordance correlation coefficient (CCC) was used to determine the agreement between the different I-ELISAs compared in this study. According to McBride (2005), CCC values below 0.9 indicate poor agreement; CCC values between 0.9 and 0.95 indicate moderate agreement; CCC values between 0.95 and 0.99 indicate substantial agreement; and CCC values above 0.99 indicate near perfect agreement. A Pearson's correlation coefficient (r) value of 1 indicates a perfect positive linear correlation between two variables, while a value of 0 indicates

no correlation (Sedgwick, 2012). There was excellent agreement between the commercial MARV GP-based I-ELISA (Integrated BioTherapeutics) and the MARV GP I-ELISA developed in this study (CCC – 0.98; Pearson's r (precision) – 0.98; bias correction factor (C_b) (accuracy) – 0.99), with a difference in qualitative results occurring in only 2/652 specimens (0.3%) (one specimen negative by the commercial MARV GP I-ELISA but low positive by the MARV GP I-ELISA developed in this study, and one low positive by the commercial MARV GP I-ELISA but negative by the MARV GP I-ELISA developed in this study). In contrast, the agreement between the commercial MARV GP-based I-ELISA and the MARV NP I-ELISA developed in this study was poor (CCC – 0.78; Pearson's r – 0.79; C_b – 0.98), with a difference in qualitative results occurring in 18/652 specimens (2.8%). Of these 18 specimens, 13 (72%) were positive for MARV GP IgG antibodies but not for MARV NP IgG antibodies, and five (28%) were positive for MARV NP IgG antibodies but not for MARV GP IgG antibodies. Not surprisingly, the agreement between the MARV GP- and NP-based I-ELISAs developed in this study was similarly poor (CCC – 0.76; Pearson's r – 0.78; C_b – 0.98), and the differences in qualitative results were identical.

2.3.3.9 Predictive values of positive and negative test results

The apparent prevalence of MARV infection in the 480 wild-caught ERBs tested in this study was 40% and 39% by the recombinant MARV GP- and NP-based I-ELISAs, respectively. Based on the diagnostic sensitivity and specificity values calculated in section 2.2.4.9 and shown in Tables 2.5 and 2.6, the PPVs for the recombinant MARV GP- and NP-based I-ELISA were 100% and 95.2%, respectively, while the NPVs for the MARV GP- and NP-based I-ELISA were 99.3% and 97.6%, respectively.

2.3.3.10 Antibody dynamics to the Marburg virus nucleoprotein and glycoprotein in experimentally infected Egyptian rousette bats

In ERBs experimentally infected with MARV, IgG antibodies against the GP became detectable as early as 5 days p.i., with the majority of bats (10/14) seroconverting at day 9 p.i. (range: 5 - 14 days p.i.) (Figure 2.12). Immunoglobulin G antibodies to the MARV GP peaked at a mean of 12 days p.i. (range: 9 - 14 days p.i.) and then started to decline towards day 42 p.i. Peak

levels of IgG antibodies to MARV GP ranged from 30.81 PP to 172.8 PP. Immunoglobulin G antibodies to the MARV NP became detectable in all bats only from day 9 p.i., with a peak in anti-MARV NP IgG levels occurring at a mean of 12 days p.i. (range: 9 - 28 days p.i.). Antibody responses to the MARV NP were more pronounced compared to MARV GP, with peak levels of IgG antibodies to MARV NP ranging from 107.4 PP to 171.1 PP. After peaking, anti-MARV NP antibody levels followed a similar decline towards day 42 p.i as anti-MARV GP antibody levels.

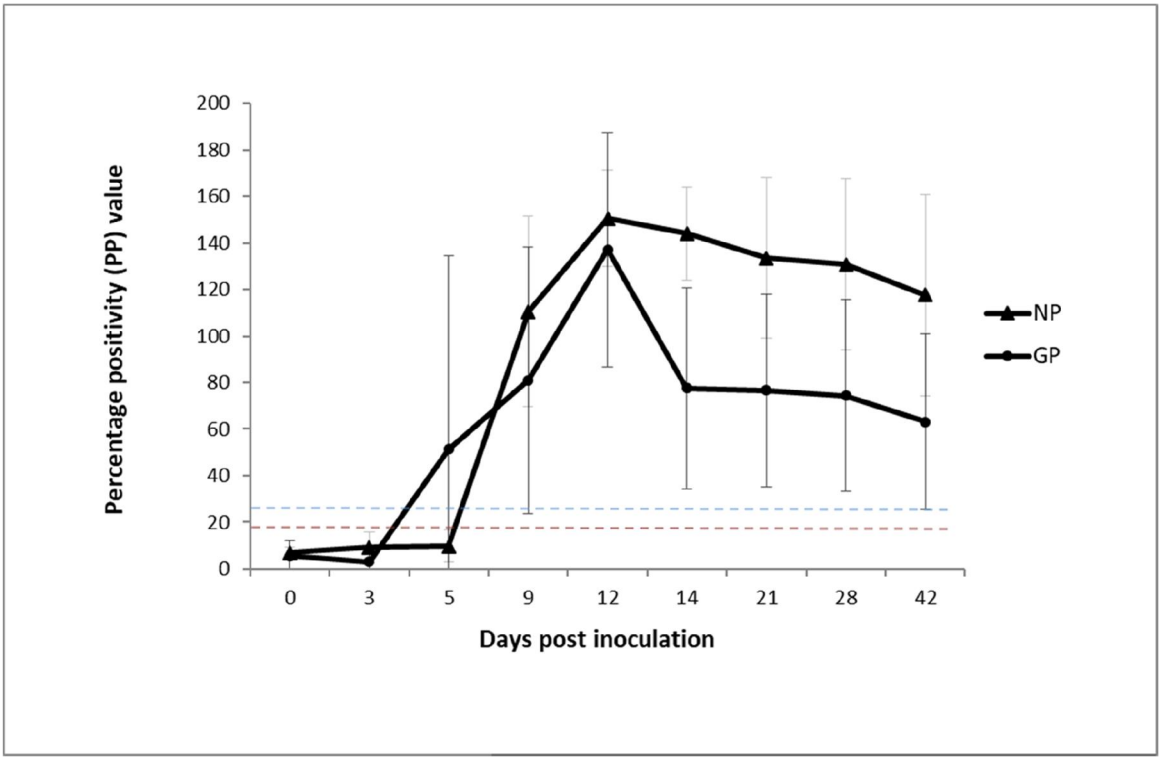


Figure 2.12: Dynamics of the humoral immune responses of Egyptian rousette bats to the glycoprotein (GP) and nucleoprotein (NP) of Marburg virus. Results are shown as the percentage positivity (PP) in relation to the positive control serum, with error bars representing the standard deviation of the measurements. The blue dashed line represents the cut-off value of the MARV NP I-ELISA at 25.7 PP, and the red dashed line represents the cut-off value of the MARV GP I-ELISA at 17.1 PP.

There was a statistically significant difference between the IgG antibody levels of experimentally infected bats to the MARV GP and MARV NP over the course of 42 days p.i. (two-tailed p-value: 0.008). There were no statistically significant differences between the IgG antibody levels of male and female bats (two-tailed p-value: 0.08), or between younger (<1 year) and older (>1 year) bats (two-tailed p-value: 0.6).

2.4 Discussion

Large outbreaks of filovirus disease have caught the world off guard during the past two decades (Towner *et al*., 2006; Baize *et al*., 2014). The unpreparedness for large filovirus outbreaks has resulted in massive chains of transmission in the human population followed by hundreds to thousands of deaths (World Health Organisation, 2016). Surveillance for filoviruses in reservoir host populations is an important aspect of predicting when and where outbreaks of filovirus disease may occur and may assist in outbreak prevention and preparedness. The role of the ERB as a reservoir host for MARV has been demonstrated (Swanepoel *et al*., 2007; Towner *et al*., 2007; Towner *et al*., 2009; Amman *et al*., 2012; Paweska *et al*., 2012), therefore there is a need for serological assays that can rapidly, safely and accurately detect IgG antibodies to the virus in bat serum. An I-ELISA described by Ksiazek and colleagues (1999) based on inactivated whole virus antigen is used for MARV surveillance and diagnostics worldwide; however, preparation of the antigen is limited to high biocontainment facilities, and ELISAs based on these antigens are prone to high background and false positive results (Rao *et al*., 1997). Recombinant protein antigens are non-infectious and may be prepared in lower biocontainment laboratories, offering a safer and more reliable alternative for use in I-ELISAs to support MARV surveillance programmes.

A few recombinant protein-based ELISAs have been described in the literature for MARV (Saijo *et al*., 2001; Nakayama *et al*., 2010; Huang *et al*., 2014) and several in-house ELISAs based on recombinant MARV proteins are also in use. The majority of these make use of recombinant antigens that have been prepared in bacterial or baculovirus-insect expression systems. Antigens prepared in bacterial expression systems are often biologically dysfunctional due to improper molecular protein folding and may be prone to high background when used in ELISA format (Khalil *et al*., 1990; Sobarzo *et al*., 2012). Due to the nature of the habitat of

bats, it is possible that bats may have a high titer of existing antibodies to *E. coli* bacteria, and minor contaminants remaining in purified recombinant proteins expressed in such systems may therefore be recognised in the bat serum, leading to high background and false positive results. While baculovirus-insect protein expression systems are capable of molecular folding and post-translational modifications, a difference in the protein glycosylation pathways between mammalian and insect cells may considerably affect the antigenic properties of the expressed recombinant proteins (Jarvis *et al*., 1998; Nakayama *et al*., 2010). In addition to the concerns mentioned above, no ELISA has been validated or evaluated for their suitability for use with bat serum, which makes the interpretation and comparison of results between different laboratories difficult.

In this study, two I-ELISAs based on the recombinant GP and NP of MARV were developed and evaluated for the detection of anti-MARV IgG in ERB sera. Both the recombinant MARV NP and GP were successfully produced in mammalian HEK 293T cells using the pCAGGS-MCS expression vector. The recombinant MARV NP antigen was considerably easier and less expensive to produce than the recombinant MARV GP antigen using the methods described in this chapter. A dynamin-dependent cellular trafficking pathway activated by filoviral GP (Sullivan *et al*., 2005), as well as a conserved, highly glycosylated mucin-like domain in the GP of filoviruses has previously been observed to cause cytotoxicity in host cells *in vitro* and *in vivo* (Takada *et al*., 2000; Yang *et al*., 2000; Simmons *et al*., 2002; Francica *et al*., 2009). For this reason, large-scale production of MARV GP is necessary in order to obtain a sufficiently high antigen yield. The mucin-like domain was not removed from the GP in this study, as previous studies have shown that a large proportion of antibodies to MARV are directed against epitopes in this region (Wilson *et al*.; 2000; Dowling *et al*., 2007; Natesan *et al*., 2016). Despite the difficulty in the production of MARV GP, both the MARV GP and NP I-ELISAs were found to be highly robust and repeatable, and could clearly distinguish between MARV-positive and -negative ERB serum with little background. The inter-laboratory reproducibility of these assays, however, remains to be evaluated.

Analytical specificity results from this study indicated no cross-reactivity from infection with a closely related filovirus (EBOV) using the MARV GP I-ELISA, demonstrating that the GP of filoviruses is highly species specific. This observation is consistent with the heterogeneous

nature of EBOV and MARV GPs (Sanchez *et al*., 1998) as well as with results from previous studies (Takada *et al*., 2007b; Nakayama *et al*., 2010). Some cross-reactivity was observed in EBOV IgG antibody-positive sera tested with the MARV NP I-ELISA. The slight cross-reactivity between the MARV NP antigen and the sera from experimentally EBOV-infected bats was not unanticipated, as the amino acid sequences of the N-terminal halves of the NPs of ebola- and marburgviruses have been shown to be highly similar (Sanchez *et al*., 1992). Furthermore, ebola- and marburgviruses share at least one conserved antibody epitope in their C-terminal halves (Ali & Islam, 2015), and cross-reactivity between the NPs of different filovirus species has been noted in other studies (Saijo *et al.*, 2001a; Natesan *et al*., 2016). The applicability of the recombinant NP antigen for use broadly in the detection of antibodies to filoviruses needs further investigation. Regrettably, sera from bats with other related infections were not available for testing.

The performance of the two I-ELISAs developed in this study was compared to an I-ELISA based on commercially available recombinant MARV GP. Ideally, the performance of new serological assays should be compared to a gold standard reference test such as a virus neutralisation assay. Likewise, clinical serum panels for the evaluation of new serological assays should be characterised using a virus neutralisation test (VNT). However, bats are known to produce limited neutralising antibodies to filoviruses (Paweska *et al*., 2012), and bat sera are often toxic at low dilutions (personal observation), making VNTs difficult to apply in filovirus bat serology. The use of sera from bats proven to be experimentally infected with MARV, along with comparison of the new assays with an I-ELISA based on a commercially available antigen, was therefore a suitable surrogate for this study. While results found in sera using the I-ELISAs based on the GP protein were comparable, the agreement between the GP-based I-ELISAs and the NP-based I-ELISA was poor. The poor agreement between the MARV GP-based I-ELISAs and the NP-based I-ELISA is not unexpected, as previous studies have indicated that humans may not consistently develop antibodies to all filovirus proteins (Groen *et al*., 2003; Sobarzo *et al*., 2012; Sobarzo *et al*., 2013; Bequart *et al*., 2014; Stonier *et al*., 2017). In this study, it was shown that antibody levels to MARV NP were of a higher magnitude than antibody levels to MARV GP in bats that do develop them. It is therefore possible that, in field-sampled bats positive for MARV NP antibodies but not for MARV GP antibodies, antibodies to the GP had already become undetectable. In addition, results of this study have shown that antibodies to

the MARV GP become detectable 4 days earlier in bats than antibodies to the MARV NP. It is therefore also possible that, in field sampled bats positive for MARV GP but not for MARV NP, MARV infection was at an early stage and antibodies to the MARV NP have not yet become detectable. Unfortunately, the dates of exposure of these bats in the wild are unknown.

Results of the I-ELISAs developed in this study indicate high estimates of diagnostic specificities (100% for the MARV GP-based I-ELISA and 96.9% for the MARV NP-based I-ELISA) compared to an I-ELISA based on commercially available recombinant MARV GP antigen (Paweska *et al*., 2015). Estimates of diagnostic sensitivity of the assays varied between 96.3% (MARV NP-based I-ELISA) and 98.8% (MARV GP-based I-ELISA). Unfortunately, serum panels for estimating the diagnostic sensitivity and specificity of each I-ELISA were limited to small numbers of bats experimentally infected with MARV in previous studies (Paweska *et al*., 2015; chapter 3 of this thesis) or juvenile bats that were brought into captivity and had lost maternal immunity to MARV. The use of experimentally infected animals in estimating diagnostic sensitivity and specificity is not ideal, as experimental infection may elicit antibody responses that are atypical of natural infection due to possible differences in virus dose and route of exposure. However, obtaining sera from actively infected individuals in the wild is notoriously difficult (Amman *et al*., 2012; Paweska *et al*., 2018) and was not possible in the current study. According to OIE guidelines, at least 300 specimens from known infected animals and 1 000 specimens from known uninfected animals should be tested to obtain accurate initial estimates of diagnostic sensitivity and specificity of serological assays, respectively (Jacobsen *et al*., 1998). While this number is unrealistic in the current context due to the difficulty of isolating virus or detecting actively infected bats in the wild by qRT-PCR, estimates of diagnostic sensitivity and specificity should continuously be updated as more well-defined sera become available from ecological and experimental MARV infection studies. Based on the estimates for diagnostic sensitivity and specificity obtained for the I-ELISAs in this study, these assays may provisionally be recognised as suitable methods for MARV surveillance in bat populations. The excellent PPV and NPV of each assay indicate the ability of each test to accurately identify true positive and negative animals, and provide further confidence in the performance of the assays.

Immunological studies in reservoir hosts may assist in identifying features of an immune response against a virus that are imperative for a protective phenotype within their natural environment, and in identifying which responses could be responsible for an increase or decrease in virus replication (Bean *et al*., 2013). Viral proteins play an important role in virus-host interactions during MARV infection (Leroy *et al*., 2002), but the kinetics of the antibody responses to the major MARV proteins in ERBs is currently unknown. The GP of MARV is important for viral entry (Mittler *et al*., 2011) and is the primary target for protective neutralising antibodies to MARV (Maruyama *et al*., 1999; Wilson *et al*., 2000; Takada *et al*., 2007a; Takada *et al*., 2007b; Bale *et al*., 2012b), while the NP drives nucleocapsid formation, is one of the most abundant proteins in a MARV-infected cell and exhibits strong antigenic properties (Saijo *et al*., 2001a; Changula *et al*., 2013). Furthermore, while GP is the most variable filoviral protein, NP is one of the most conserved (Natesan *et al*., 2016). This study has indicated that the I-ELISAs developed in this study are suitable methods to evaluate the antibody kinetics of ERBs in response to MARV infection. Results showed a peak in both anti-MARV NP (range: 9-28 days p.i.) and anti-MARV GP (range: 9-14 days p.i.) antibody levels in experimentally infected ERBs at day 12 p.i. Similarly, a previous study by Paweska and colleagues (2015) showed a peak in anti-MARV GP IgG antibody levels in experimentally infected bats at day 14 p.i. (range: 9-21 days p.i.). A study by Schuh and colleagues (2017a) showed a later peak in anti-MARV NP IgG antibodies, which occurred at a mean of 20 days p.i. (range: 14-28 days p.i.). The peak ranges of anti-MARV GP and anti-MARV NP antibodies in this study and the study by Paweska and colleagues (2015) overlap with reported periods when viraemia becomes undetectable and the virus is cleared from bat tissues (12-16 days p.i.) (Paweska *et al*., 2012; Paweska *et al.*, 2015; Schuh *et al*., 2017a). In this study, seroconversion occurred slightly faster for MARV GP than for MARV NP; however, the antibody response to MARV NP was significantly more pronounced. This result is similar to results found in an investigation of the antibody repertoire of humans following non-fatal MARV infection (Natesan *et al*., 2016). The stronger antibody response to the NP of MARV may possibly be explained by the high abundance of NP in the infected cell compared to MARV GP (Baker *et al*., 2016). In addition, MARV GP glycosylation may shield antibody epitopes on the protein (Francica *et al*., 2010), which might explain the weaker anti-MARV GP antibody response.

In conclusion, the I-ELISAs developed in this study are safe and suitable test methods for the detection of anti-MARV IgG antibodies in bat sera and may be employed in future serosurveillance studies in bat populations in both MARV-endemic and non-endemic countries. Although a full validation of the assays developed in this study was not possible, results obtained indicate a high diagnostic sensitivity and specificity for each assay, with the MARV GP-based I-ELISA demonstrating superior performance. The I-ELISAs are also useful tools for the characterisation of antibody responses to MARV in reservoir hosts and may be employed in studies evaluating the role of antibody responses in protection against MARV infection.

CHAPTER 3: ANTIBODY RESPONSES OF EGYPTIAN ROUSETTE BATS TO MARBURG VIRUS AND THEIR ROLE IN PROTECTION AGAINST INFECTION

Published* as:

Storm N, Jansen van Vuren P, Markotter W and Paweska JT (2018). Antibody responses to Marburg virus in Egyptian rousette bats and their role in protection against infection. *Viruses* **10**: 73.

*Elaborations on the methods and discussion, and results for juvenile bat measurements are presented in this chapter, which was not included in the publication due to journal restrictions.

Partially presented at international conferences as:

Storm N, Jansen van Vuren P, Markotter W and Paweska JT. The role of passive and acquired humoral immunity in the maintenance of Marburg virus in *Rousettus aegyptiacus* bat colonies. The 9th International Filovirus Symposium, Marburg, Germany, September 13-16, 2017 (Oral presentation)

Storm N, Jansen van Vuren P, Markotter W and Paweska JT. Humoral immune responses of *Rousettus aegyptiacus* to Marburg virus. The 4th International One Health Congress & 6th Biennial Congress of the International Association for Ecology and Health, Melbourne, Australia, December 3-7, 2016 (Oral presentation).

3.1 Introduction

Marburg virus (MARV) causes severe and often fatal haemorrhagic fever in humans and non-human primates (MacNeil *et al*., 2010). The Egyptian rousette bat (ERB), *Rousettus aegyptiacus*, has been recognized as a reservoir host for MARV based on repeated RNA and anti-MARV antibody detection in (Swanepoel *et al*., 2007; Towner *et al*., 2007; Paweska *et al*., 2018) and isolation of the virus from naturally infected bats (Towner *et al*., 2009; Amman *et al*., 2012; Amman *et al*., 2015), and the absence of clinical disease following experimental inoculation (Paweska *et al*., 2012; Amman *et al*., 2015; Paweska *et al*., 2015; Schuh *et al*.,

2017a) coupled with viral shedding (Amman *et al*., 2015; Schuh *et al*., 2017a). Despite this progress made in identifying and studying the reservoir host for MARV, knowledge of the biology of the virus in ERBs remains sparse. Spillover of MARV into human and animal populations appear to coincide with periods of increased viral shedding from ERBs (Amman *et al*., 2012), but the mechanisms driving the transmission and maintenance of MARV, including the natural ports of entry and exit in this bat species, remain to be described. At present, three hypotheses for MARV transmission dynamics in ERB populations exist: 1) bats may obtain life-long immunity following recovery from a primary infection, and new outbreaks of the virus only occur when the pool of susceptible bats is replenished by weaned juveniles that have lost maternal immunity; 2) immunity to MARV in bats may be transient, with the virus being able to persist through fluctuating herd immunity; or 3) bats may be persistently infected with MARV, shedding virus periodically due to physiological or environmental stress factors (Plowright *et al*., 2016). Mathematical models and longitudinal ecological studies of filoviruses in ERBs have suggested that a seasonal increase in viral shedding may occur as a result of waning immunity, births and migration (Amman *et al*., 2012; Peel *et al*., 2014; Hayman *et al*., 2015). At present, the only evidence available points to the loss of maternal immunity in juvenile bats as being a major driver of MARV maintenance in nature (Amman *et al*., 2012), but information on exactly when juveniles lose maternal immunity and become susceptible to infection with the virus is limited.

Little is known about the role of antibody responses in the protection of ERBs against MARV infection. It is also unclear whether primary infection with MARV results in long-term or transient protective immunity. An experimental study showed that antibodies against MARV in ERBs declined to undetectable levels by the third month post-infection (Schuh *et al*., 2017a), making them potentially susceptible to reinfection. Antibodies may not be a major driver of viral clearance in bats (Middleton *et al*., 2007; Halpin *et al*., 2012). For example, in fruit bats experimentally infected with henipavirus, some individuals continued to shed virus despite detectable antibody titers (Middleton *et al*., 2007; Halpin *et al*., 2011). In addition, a study by Nakayama and colleagues (2011) suggested that antibodies might even enhance MARV infection *in vitro*.

Determining the duration of actively and passively acquired immunity in ERBs, and whether actively acquired anti-MARV antibodies in ERBs are protective against reinfection, may assist in understanding how herd immunity influences MARV maintenance and population transmission dynamics. This knowledge may assist in predicting and preventing spillover events into human and other animal populations. Furthermore, age-based analyses of MARV-seroprevalence may assist in more accurately determining the incidence of MARV in an ERB population, as the presence of maternal immunoglobulin G (IgG) antibodies may make results of serosurveillance studies difficult to interpret.

In this chapter, it is shown that maternal antibodies to MARV are lost in juvenile ERBs between 4 and 5 months after birth, thus making them potentially susceptible to infection with the virus. Actively acquired antibodies to MARV in ERBs following experimental or natural infection remained detectable in the majority of bats at 110 days post-infection (67%) and 11 months after capture (84%), respectively, contrasting with the results found by Schuh and colleagues (2017a). A previous study demonstrated protective immunity against MARV reinfection and replication in ERBs when reinoculated 48 days after infection (Paweska *et al*., 2015). Results of the current study indicate that IgG antibodies do not completely protect previously MARV-exposed bats against reinfection, but appear to prevent systemic spread of the virus. The resulting lack of viral shedding implies that reinfection of previously exposed bats is not a major contributor to the transmission and maintenance dynamics of MARV in ERBs in nature.

3.2 Materials and methods

3.2.1 Regulatory requirements and ethics clearance

A permit to capture ERBs in the Limpopo Province of South Africa was obtained from the Limpopo Department of Economic Development, Environment and Tourism (CPM006806; Appendix A) as well as the Gauteng Department of Agriculture and Rural Development (CPB6003767; Appendix A). Approval for establishing an ERB breeding colony and performing experimental infections of ERBs with MARV was obtained from the Department of Agriculture, Forestry and Fisheries of South Africa (12/11/1/1/13; Appendix A). Ethics approval for the colonisation and experimental infection of ERBs with MARV was acquired from the National Health Laboratory Service Animal Ethics Committee (AEC 136/12, AEC

139/13; Appendix A), as well as the University of Pretoria Animal Ethics Committee (EC056-14, H018-16; Appendix A).

3.2.2 Experiment 1: Duration of maternal immunity to Marburg virus in juvenile Egyptian rousette bats

Twenty-six juvenile ERBs were captured at a cave in the Matlapitsi Valley in the Limpopo Province of South Africa using harp traps. Upon capture, juvenile status was confirmed by observing a lack of epiphyseal-diaphyseal fusion of the long phalanges under backlight illumination, and juvenile size and pelage (Brunet-Rossini & Wilkinson, 2009). The bats were brought into captivity at the animal facility of the National Institute for Communicable Diseases of the National Health Laboratory Service (NICD-NHLS) in temporary cages, and relocated to a larger flight cage for colonisation as described in a previous study (Paweska *et al*., 2012). The bats were bled by cardiac puncture under anaesthesia (35 mg/kg body mass Anaket-V (Bayer) and 5 mg/kg body mass Rompum (Bayer) diluted in sterile Dulbecco's phosphate buffered saline (DPBS; Lonza) and given by intramuscular injection) on four separate occasions. At the time of first sampling, the bats were weighed and their forearms measured using a vernier caliper (dialMax, Wiha Tools Ltd.). The age of each bat was estimated according to the forearm length growth curve published by Mutere (1968).

In addition to wild-caught juvenile bats, 20 bats born in captivity to MARV-seropositive dams were bled on five separate occasions, with sampling commencing at 3 months of age. Sera were separated from the blood samples by centrifugation at 3 000 x *g* for 10 minutes (Eppendorf MiniSpin, Merck) and were tested for anti-MARV IgG antibodies using a MARV glycoprotein (GP)-based indirect enzyme-linked immunosorbent assay (I-ELISA) as described in section 2.2.1.2. The negative control serum, conjugate controls and test sera were assayed in duplicate at a dilution of 1:100, and positive control serum was assayed in quadruplicate. Positive control serum was derived from a pool of sera from ERBs infected with MARV during a previous experiment (Paweska *et al*., 2012; Appendix B), and negative control serum was derived from a pool of serum obtained from six MARV-naive ERBs born in captivity (Appendix B). Optical density (OD) values were measured at 405 nm using a microplate reader. The means of the OD

values of the test sera replicates were calculated and converted to a percentage positivity (PP) relative to the positive control serum using the following equation (Paweska *et al*., 2005):

$$Percentage\ positivity\ = \frac{Mean\ net\ OD\ of\ test\ sera\ replicates}{Mean\ net\ OD\ of\ positive\ control\ serum\ replicates} x\ 100$$

3.2.3 Experiment 2: Duration of the antibody response to Marburg virus in experimentally infected Egyptian rousette bats

Six MARV-naive ERBs were inoculated subcutaneously with 100 µl of tissue culture supernatant containing $10^{5.3}$/ml tissue culture infectious dose ($TCID_{50}$) of MARV/Hsap/COD/99/Watsa-SPU148-99-I (second passage in Vero cells). The bats were clinically monitored and bled over a period of 110 days as described in section 3.2.2. Sera were tested for anti-MARV GP IgG antibodies using I-ELISA as described in section 2.2.4.2.

3.2.4 Experiment 3: Duration of the antibody response to Marburg virus in naturally infected Egyptian rousette bats

Thirty-eight bats that had previously been exposed to MARV in nature as evidenced by the presence of anti-MARV IgG antibodies in sera at the time of capture (PP value range: 22.6–176.1; cut-off value for I-ELISA: 17.1 PP) were brought into captivity as described in 3.2.2. The bats were monitored for the presence of anti-MARV IgG antibodies in their sera over a period of 11 months using a recombinant MARV GP-based I-ELISA as described in 2.2.4.2.

3.2.5 Experiment 4: Reinfection of seropositive Egyptian rousette bats with Marburg virus

Seventeen wild-caught bats with MARV GP-specific IgG PP values ranging from 26.5 to 146.3 were selected for this experiment. Bat sera were tested for the presence of IgG antibodies to MARV at both capture and one week prior to commencing the experiment. The infection histories of the bats were unknown, however, the bats had most likely been infected with the

MARV variant circulating in a cave located in the Matlapitsi Valley, Limpopo Province, South Africa (Paweska *et al.*, 2018), where the bats were captured. Fifteen bats (13 adult females and two adult males) were inoculated subcutaneously with 100 µl of tissue culture supernatant containing $10^{5.3}$/ml $TCID_{50}$ of MARV/Hsap/COD/99/Watsa-SPU148-99-I (second passage in Vero cells). Two control bats (adult females) were inoculated subcutaneously with 100 µl of Eagle's Minimum Essential Medium (EMEM; Lonza). Bats were clinically monitored daily, and bled, serially euthanised by cardiac exsanguination under anaesthesia and dissected on days 0, 3, 5, 7, 9 and 12 post inoculation (p.i). In addition, oral, nasal, rectal, penile and vaginal swabs were collected into 500 µl EMEM using sterile cotton swabs.

3.2.5.1 Real-time quantitative reverse transcription polymerase chain reaction

Bat tissues were homogenised in EMEM (Lonza) as 10% (w/v) suspensions using the Tissuelyser II and 5 mm sterile stainless steel beads (QIAGEN) at 30 Hz for 8 minutes. Homogenates were centrifuged (Eppendorf MiniSpin, Merck) at 12 225 x *g* for 5 minutes. Ribonucleic acid was extracted from serum, swabs and the supernatant of the 10% tissue homogenates in EMEM (Lonza) using the QIAamp Viral RNA Mini kit (QIAGEN) according to the manufacturer's instructions. Briefly, 140 µl of serum, clarified tissue homogenate or swab suspension was added to a microcentrifuge tube with 560 µl AVL lysis buffer containing 5.6 µl carrier RNA (1 µg/µl). The contents of the tube was mixed for 15 seconds and incubated at 25°C for 10 minutes. A volume of 560 µl 96% ethanol (Merck) was added and the contents mixed for 15 seconds. Six hundred and thirty microliters of the solution was added to a QIAamp Mini column in a collection tube. The tube was centrifuged at 8 000 x *g* for 1 minute (Eppendorf MiniSpin, Merck). The filtrate was discarded and the previous step repeated. Five hundred microliters of buffer AW1 was added and the tube centrifuged at 8 000 x *g* for 1 minute (Eppendorf Mini Spin, Merck). The filtrate was discarded and 500 µl of buffer AW2 was added. The tube was centrifuged at 12 100 x *g* for 3 minutes (Eppendorf Mini Spin, Merck). The QIAamp Mini column was placed into a sterile microcentrifuge tube and centrifuged at 12 100 x *g* for 1 minute (Eppendorf Mini Spin, Merck). After placing the column into a clean microcentrifuge tube, 60 µl of buffer AVE was added. The tube was incubated at 25°C for 1 minute, followed by centrifugation at 8 000 x *g* for 1 minute (Eppendorf Mini Spin, Merck). The RNA was stored at -70°C until required.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the Qiagen One-Step RT-PCR kit (Qiagen) according to the manufacturer's instructions. Briefly, 5 µl of RNA was added to a master mix containing 1 x Qiagen One-Step RT-PCR buffer with 12.5 mM magnesium chloride, 0.4 mM deoxyribonucleotide triphosphate mix, 0.6 µM forward primer (Filo A2.3, Panning *et al*., 2007), 0.7 µM reverse primer (Filo B-Ra, Panning *et al*., 2007), 0.1 µM probe (FAMMBG, Panning *et al*., 2007), 40 µg/ml bovine serum albumin, 2 µl Qiagen One-Step RT-PCR enzyme and deionised water to a volume of 20 µl. The MARV L gene was amplified using the following cycling conditions (Lightcycler 480, Roche): reverse transcription at 50°C for 30 minutes, followed by a hot start Taq activation step of 95°C for 15 seconds, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 52°C for 25 seconds and extension at 72°C for 20 seconds. Ribonucleic acid copy numbers per reaction were converted to copy numbers per gram tissue or per millilitre serum, followed by conversion into $TCID_{50}$ equivalents using a logarithmic titration curve as previously described (Paweska *et al*., 2012).

3.2.5.2 Virus isolation

Virus isolation was attempted on all specimens that tested positive by qRT-PCR. Vero E6 cells were cultured to confluency in EMEM (Lonza) containing 10% foetal bovine serum (FBS; HyClone) in 25 cm^2 flasks (Porvair). After removal of the supernatant, the flasks were inoculated with 500 µl of specimen and incubated for 1 hour at 37°C. The inoculums were removed and the flasks rinsed with DPBS (Lonza). Fresh EMEM containing 2% FBS was added to each flask, and the flasks were incubated at 37°C for 14 days. The medium was changed at 7 days p.i. Aliquots of the cell culture supernatant were collected on the day of inoculation, as well as on days 3, 7, 11 and 14 p.i. The aliquots were tested for MARV replication using qRT-PCR described in section 3.2.5.1.

3.2.5.3 Virus neutralisation index

In order to evaluate the ability of anti-MARV IgG in sera from bats naturally exposed to a local strain of MARV (see section 3.2.5) to neutralise a genetically distinct MARV strain, a virus

neutralisation index test was performed. Briefly, ten-fold dilutions of MARV/Hsap/COD/99/Watsa-SPU148-99-I (second passage in Vero cells), and MARV/Hsap/ZAF/75/Ozolin (fourth passage in Vero cells), were prepared in 96 well cell culture plates (NUNC) in sextuplicate. Triplicates of each ten-fold dilution were mixed with a 1:20 dilution of pooled MARV-positive or -negative bat serum (Appendix B) in Minimum Essential Medium (MEM) Rega-3 (Gibco, Thermo Fisher Scientific) and the plate was incubated at 37°C for 1 hour. A confluent flask of human adrenal carcinoma cells (SW-13) was trypsinised into 40 ml MEM Rega-3 containing 8% FBS (HyClone). One hundred microliters of the cell suspension was added to each well of the plate, and the plate was incubated at 37°C in a 5% CO_2 atmosphere for 7 days. The plate was fixed in 80% acetone (Sigma-Aldrich) in distilled water, and stained with rabbit anti-MARV serum (prepared in-house) at a dilution of 1:300 followed by a 1:160 dilution of an anti-rabbit fluorescein isothiocyanate-labeled secondary antibody (3 mg/ml; Sigma-Aldrich). Fluorescent foci were observed using a fluorescence microscope (EVOS) and the viral titers determined using the method of Spearman and Kärber (Spearman, 1908; Kärber, 1931).

3.2.6 Statistical analysis

Results obtained in this experiment were compared to results obtained by Paweska and colleagues (2015) in a previous study, in which MARV-naive bats were experimentally infected with MARV. All statistical tests were performed in Microsoft Excel. Correlation between I-ELISA PP value and levels of equivalent viraemia in reinfected bats was determined using Spearman's Rank-Order Correlation. The Student's t-test was performed to determine whether anti-MARV IgG levels differed significantly between naive (Paweska *et al*., 2015) and seropositive MARV infected bats (two-tailed p-value < 0.05). The statistical significance of differences in viral load in the tissues of naive (Paweska *et al*., 2015) and seropositive infected bats was determined by performing the Kruskal-Wallis rank test.

3.3 Results

3.3.1 Experiment 1: Duration of maternal immunity to Marburg virus in juvenile Egyptian rousette bats

The average age of the juvenile ERBs at the first bleed was 8 weeks, with a range of 6 to 10 weeks (Table 3.1). Epiphyseal-diaphyseal fusion of the long phalanges was not observed in any of the bats at this point in time.

Table 3.1: Measurements and age estimates of juvenile Egyptian rousette bats at the time of first sampling

Bat number	Sex	Weight (g)	Forearm (mm)	Fusion of epiphyses	Age estimate at first bleed*
1	M	57	71.9	None	8 weeks
2	F	48	62.2	None	6 weeks
3	F	46	64.2	None	7 weeks
4	F	64	70.1	None	8 weeks
5	M	60	70.6	None	8 weeks
6	M	57	72.5	None	9 weeks
7	M	58	72.5	None	9 weeks
8	F	50	71.9	None	8 weeks
9	M	48	74.2	None	10 weeks
10	M	61	70.7	None	8 weeks
11	M	68	71.5	None	8 weeks
12	F	68	71.4	None	8 weeks
13	F	65	72.1	None	9 weeks
14	M	63	72	None	9 weeks
15	M	46	68.9	None	8 weeks
16	M	49	70.4	None	8 weeks
17	F	52	71.6	None	8 weeks
18	F	56	72.6	None	9 weeks
19	F	47	68.8	None	8 weeks
20	F	63	72.2	None	9 weeks
21	F	67	71.3	None	8 weeks
22	F	58	72.4	None	9 weeks
23	M	57	70.9	None	8 weeks
24	M	48	69.8	None	8 weeks
25	F	56	73.3	None	9 weeks
26	M	61	74.1	None	9 weeks

Abbreviations: M - Male, F - Female, g - grams, mm - millimeter

*Based on forearm length growth curve of Mutere (1968): 42.0 mm - 61.9 mm, 4 weeks ± 1 week; 62 mm - 67.9 mm; 6 weeks ± 1 week; 68.0 mm - 71.9 mm, 8 weeks ± 1 week; 72 mm - 77.9 mm, 10 weeks ± 1 week; 78 mm - 81.9 mm, 12 weeks ± 1 week

All wild-caught juveniles tested in this study were born to dams previously naturally infected with MARV as evidenced by the presence of MARV-specific maternal IgG antibodies in the juveniles (26/26) at the time of the first sampling (Figure 3.1). By the second bleed (approximately 3 months after birth), the percentage of juveniles with detectable maternal IgG antibodies to MARV had declined to 50% (13/26). At approximately 5 months after birth, maternal IgG antibodies to MARV could only be detected in a single bat, and by 7 months after birth, none of the bats had detectable levels of maternal IgG antibodies to MARV.

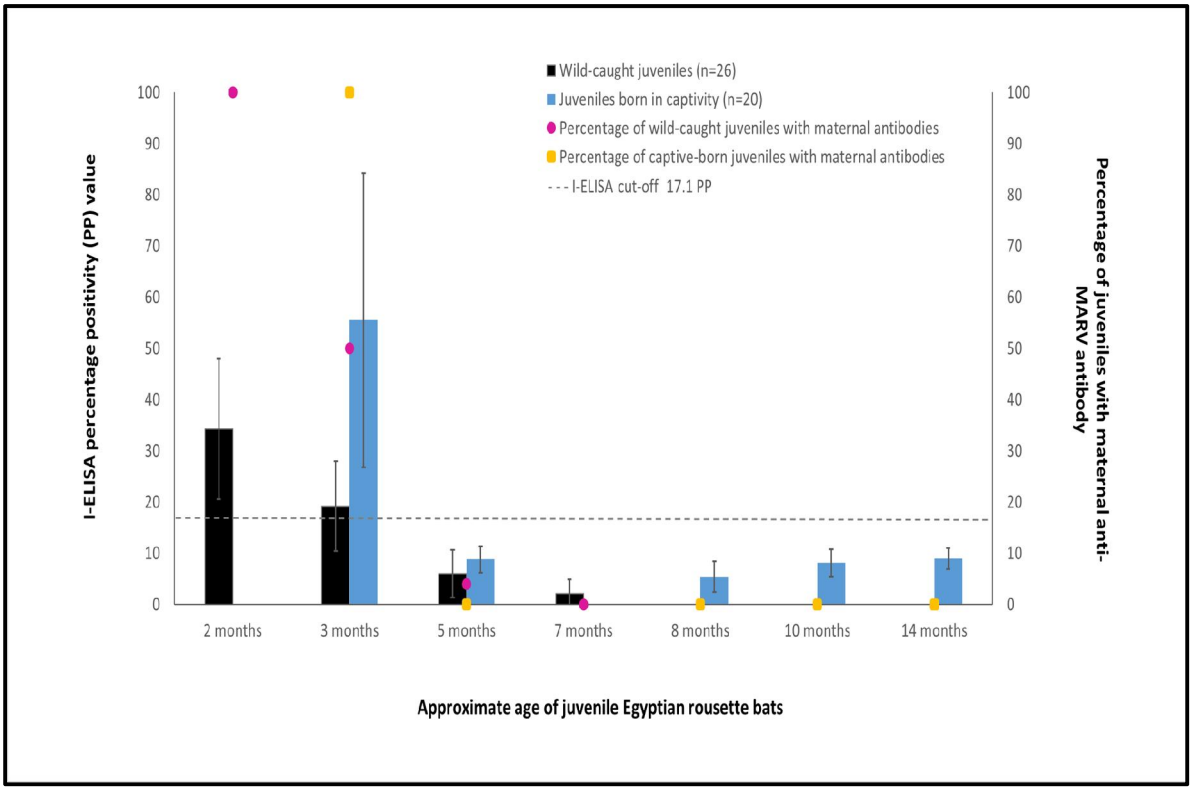


Figure 3.1: Mean maternal anti-Marburg virus (MARV) immunoglobulin G antibody levels in juvenile bats born from naturally exposed mothers, with error bars representing the standard deviation of the measurements. Results of the indirect enzyme-linked immunosorbent assay are shown as the percentage positivity (PP) in relation to the positive control serum (left-hand y-axis). The percentage of juveniles with maternal anti-MARV antibodies is displayed on the right-hand y-axis.

Similarly, no maternal antibodies could be detected in juvenile bats born from captive MARV-seropositive dams at 5 and 8 months of age, even though maternal antibody titers to MARV in these bats at 3 months of age were much higher than those of wild-caught juvenile bats at a comparable age (Figure 3.1).

3.3.2 Experiment 2: Duration of the antibody response to Marburg virus in experimentally infected Egyptian rousette bats

Immunoglobulin G antibodies to MARV peaked in all experimentally infected bats at day 14 p.i. (Figure 3.2), and then started to decline towards day 110 p.i. There was considerable variation in the immune responses of each bat, with three of the six bats producing IgG antibodies with a maximum average I-ELISA PP value of only 41.2.

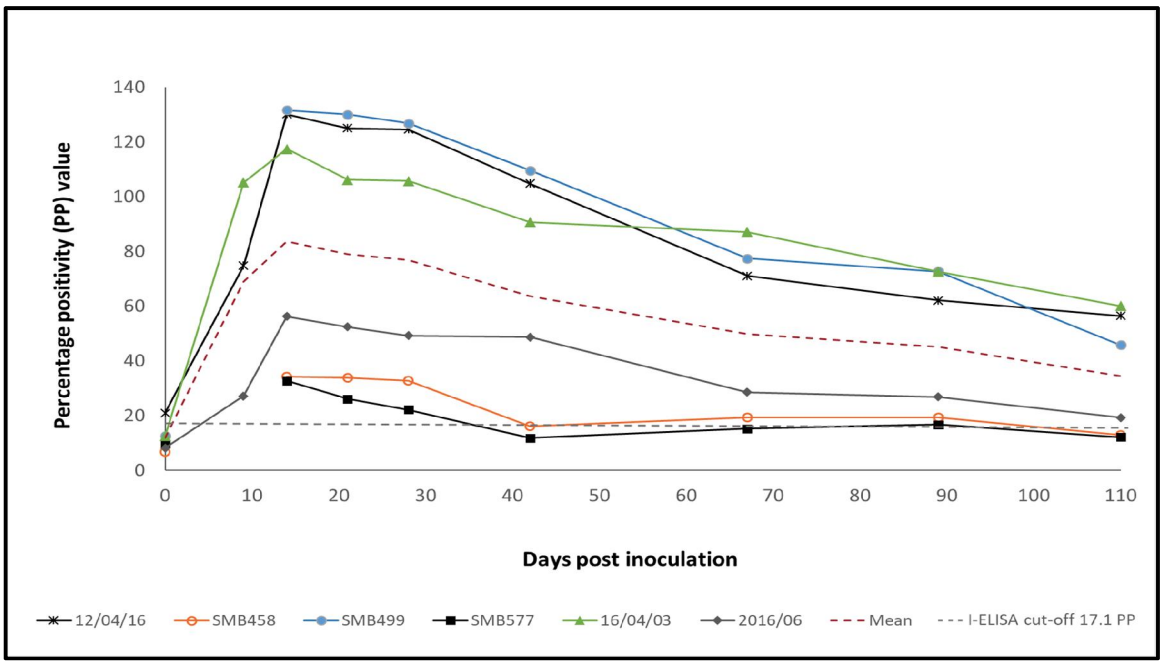


Figure 3.2: Duration of the immunoglobulin G (IgG) immune response to Marburg virus in individual experimentally infected Egyptian rousette bats (n = 6), with the dashed red line representing the mean duration of the IgG immune response. Results of the indirect enzyme-linked immunosorbent assay are shown as the percentage positivity (PP) in relation to the positive control serum.

In two of these bats, IgG antibodies declined to undetectable levels by day 110 p.i., but IgG antibodies could still be detected in four of the six bats (67%) on this day.

3.3.3 Experiment 3: Duration of the antibody response to Marburg virus in naturally infected Egyptian rousette bats

Immunoglobulin G antibodies to MARV in previously naturally exposed bats gradually declined over a period of 11 months (Figure 3.3). Marburg virus-specific antibodies became undetectable in only six of the 38 bats (15.8%) between month 9 and 11 after capture.

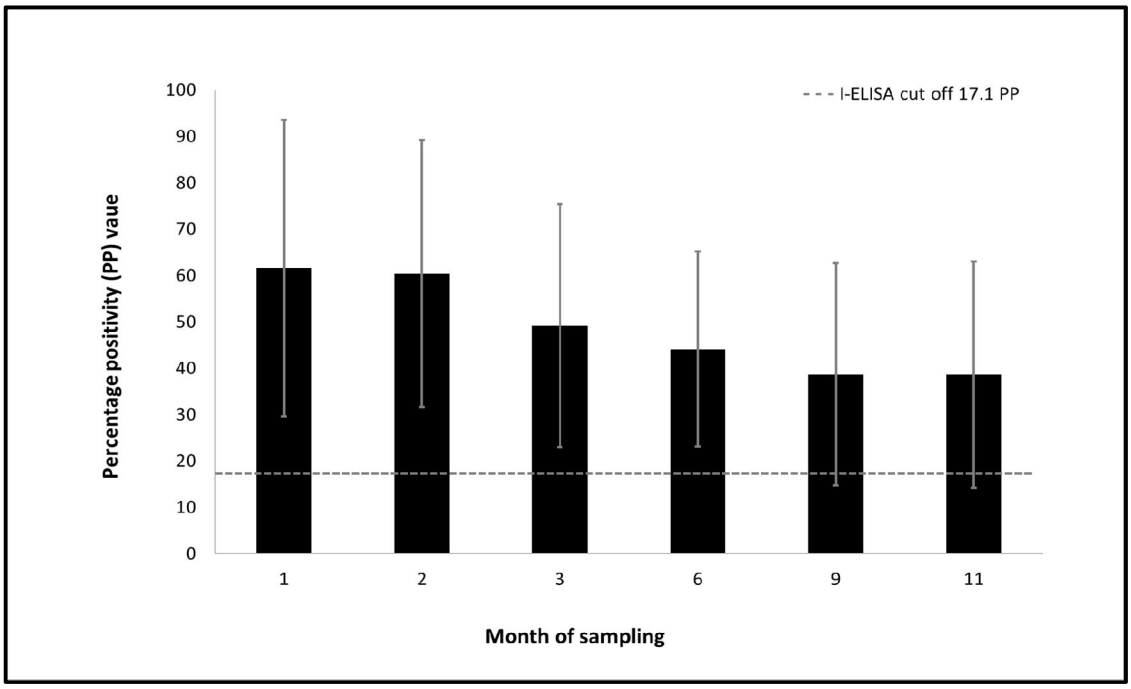


Figure 3.3: Mean duration of the immunoglobulin G immune response to Marburg virus in previously naturally exposed Egyptian rousette bats (n = 38), with error bars representing the standard deviation of the measurements. Results of the indirect enzyme-linked immunosorbent assay are shown as the percentage positivity (PP) in relation to the positive control serum.

3.3.4 Experiment 4: Reinfection of seropositive Egyptian rousette bats with Marburg virus

3.3.4.1 Serology

There were no apparent signs of morbidity or mortality in any of the MARV-inoculated or control bats for the duration of the experiment. There was a statistically significant difference between the I-ELISA PP values of naive (Paweska *et al*., 2015) and seropositive bats both before (two-tailed p-value: 0.00001), and after inoculation with MARV (two-tailed p-value: 0.0002). A substantial boosting effect of anti-MARV GP IgG levels was noted in the MARV-inoculated bats from day 5 p.i. (Figure 3.4). The anti-MARV IgG levels in control bats remained unchanged for the duration of this study.

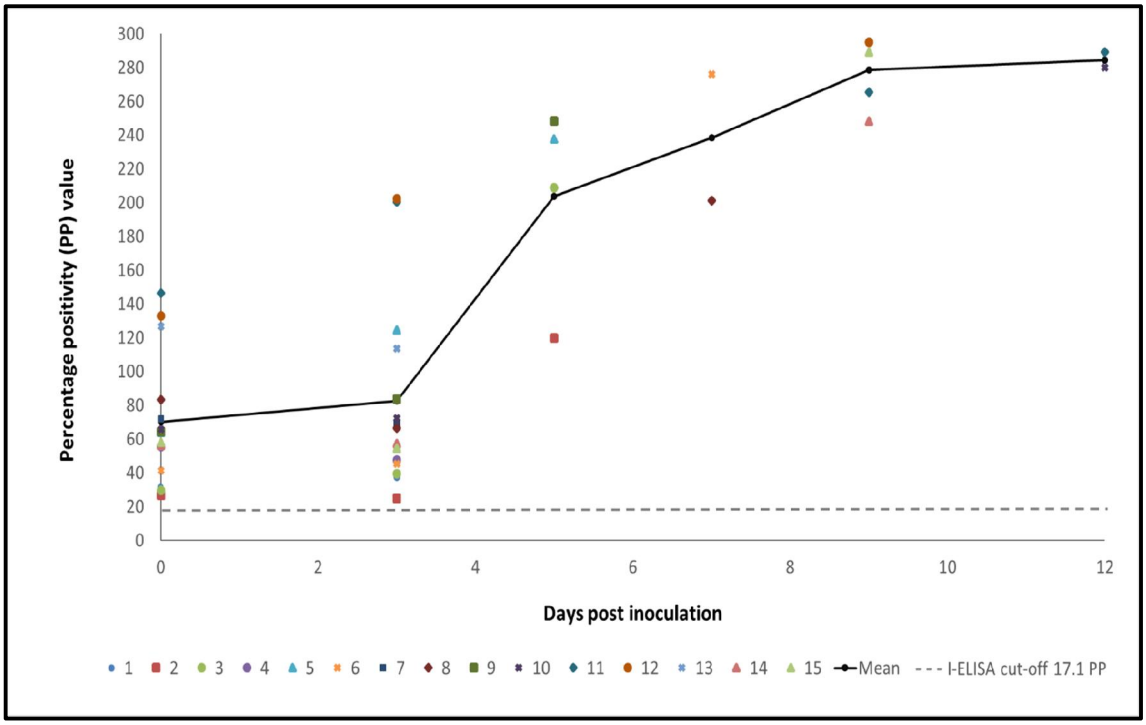


Figure 3.4: Immunoglobulin G antibody responses in 15 Egyptian rousette bats with pre-existing natural humoral immunity following experimental infection with Marburg virus. Results of the indirect enzyme-linked immunosorbent assay are shown as the percentage positivity (PP) in relation to the positive control serum.

3.3.4.2 Detection of Marburg virus RNA by real-time quantitative reverse transcription polymerase reaction and virus isolation

Based on qRT-PCR, 11 of the 15 seropositive bats (73.3%) were viraemic on the third day p.i. (Table 3.2), similar to findings in naive bats infected with MARV (Paweska *et al*., 2015). However, the challenge virus was isolated from only one qRT-PCR positive serum (bat 4, $10^{1.08}$ $TCID_{50}$/ml, I-ELISA PP value 47.45) on day 3 p.i. Replication of the virus in serum was also demonstrated in one additional bat (bat 2, I-ELISA PP value 26.54), with the level of equivalent viraemia increasing from $10^{0.7}$ $TCID_{50}$/ml on day 3 p.i. to $10^{1.38}$ $TCID_{50}$/ml on day 5 p.i. Unlike in naive infected bats (Paweska *et al.*, 2015), MARV could not be detected in the blood of seropositive bats from day 7 p.i. Marburg virus concentrations in the serum of seropositive bats on day 3 p.i. ranged from $10^{-0.09}$ $TCID_{50}$/ml to $10^{2.3}$ $TCID_{50}$/ml. In comparison, MARV concentrations in the serum of naive infected bats (Paweska *et al*., 2015) on day 3 p.i. ranged from $10^{-0.3}$ $TCID_{50}$/ml to $10^{2.1}$ $TCID_{50}$/ml, with no statistically significant differences between the levels of equivalent viraemia in naive (Paweska *et al*., 2015) and seropositive bats on this day (two-tailed p-value: 0.74).

MARV RNA was detected in the spleen of 73.3% of seropositive bats from day 3 to 12 p.i. (virus concentration range: $10^{-0.6}$ $TCID_{50}$/g tissue - $10^{2.91}$ $TCID_{50}$/g tissue), and in the liver (47%) from day 3 to 9 p.i. (virus concentration range: $10^{-0.27}$ $TCID_{50}$/g tissue - $10^{1.75}$ $TCID_{50}$/g tissue). In comparison, MARV concentrations in the spleens and livers of naive infected bats between days 3 and 12 p.i. ranged from $10^{2.95}$ $TCID_{50}$/g tissue to $10^{3.89}$ $TCID_{50}$/g tissue, and from $10^{2.6}$ $TCID_{50}$/g tissue to $10^{3.7}$ $TCID_{50}$/g tissue, respectively (Paweska *et al*., 2015). There were no significant differences between the mean MARV concentrations in the spleens (naive bats: $10^{2.96}$ $TCID_{50}$/g tissue; seropositive bats: $10^{2.57}$ $TCID_{50}$/g tissue; p-value: 0.51) and livers (naive bats: $10^{1.92}$ $TCID_{50}$/g tissue; seropositive bats: $10^{1.5}$ $TCID_{50}$/g tissue; p-value: 0.28) of naive (Paweska *et al*., 2015) and seropositive bats on day 3 p.i. However, the virus was cleared earlier in seropositive bats, with a statistically significant difference in viral loads in the livers (p-value: 0.01) and spleens (p-value: 0.02) of naive and seropositive bats from day 5 p.i.

Viral RNA was detected in the lung of one seropositive bat on day 3 p.i. ($10^{-0.47}$ $TCID_{50}$/g tissue) and in one nasal swab on day 5 p.i. ($10^{0.82}$ $TCID_{50}$/ml) (Table 3.2). No MARV RNA could be detected in any of the other tissues sampled in this study (Table 3.2).

Table 3.2: Quantitative reverse-transcription polymerase chain reaction and virus isolation results in specimens from seropositive Egyptian rousette bats experimentally inoculated with Marburg virus

	Days after Inoculation [a]				
	3 (*n* = 15)	**5 (*n* = 3)**	**7 (*n* = 3)**	**9 (*n* = 6)**	**12 (*n* = 2)**
Bat IDs	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15	2, 5, 9	3, 6, 8	10, 11, 12, 13, 14, 15	11, 15
Specimen					
Serum	11/15; VI: 1/11	1/3; VI: 0/1	0/3	0/6	0/2
Rectal swab	0/15	0/3	0/3	0/6	0/2
Nasal swab	0/15	1/3; VI: 0/1	0/3	0/6	0/2
Oral swab	0/15	0/3	0/3	0/6	0/2
Vaginal swab	NS	NS	NS	0/4	0/1
Penile swab	NS	NS	NS	0/2	0/1
Liver	2/3; VI: 0/2	3/3; VI: 0/3	0/3	1/4; VI: 0/1	0/2
Spleen	3/3; VI: 0/3	2/3; VI: 0/2	1/3; VI: 0/1	1/4; VI: 0/1	1/2; VI: 0/1
Kidney	0/3	0/3	0/3	0/4	0/2
Lung	1/3; VI: 0/1	0/3	0/3	0/4	0/2
Intestine	0/3	0/3	0/3	0/4	0/2
Stomach	0/3	0/3	0/3	0/4	0/2
Rectum	0/3	0/3	0/3	0/4	0/2
Bladder	0/3	0/3	0/3	0/4	0/2
Reproductive organs	0/3	0/3	0/3	0/4	0/2
Salivary glands	0/3	0/3	0/3	0/4	0/2

Abbreviations: ID—identification number; NS—not sampled; PCR—polymerase chain reaction; VI—virus isolation. [a] Data represents the number of positive samples/number tested. Data designates PCR results unless otherwise stated. VI was only attempted on specimens with positive PCR results.

These findings differ from results obtained from a previous study of experimental MARV infection in naive bats, where MARV RNA could be detected in the salivary glands (18% of bats), kidney (9%), intestine (27%), bladder (5%) and the reproductive tract (18%) between 3

and 12 days p.i. (Paweska *et al*., 2015). No MARV RNA was detected in any specimens collected from control bats.

There was a negative correlation ($r_s = -0.61$, Spearman's: $p = 0.001$) between the I-ELISA PP value and the level of equivalent viraemia, suggesting that ERBs are to some extent more likely to become viraemic upon reinfection when levels of MARV-specific IgG have declined.

3.3.4.3 Virus neutralisation index

The titer of MARV/Ozolin titrated on the SW-13 cells was 1.58×10^7 $TCID_{50}$/ml, and was neutralised to a titer of 1.58×10^6 $TCID_{50}$/ml by the anti-MARV/Matlapitsi antibody positive bat serum. The titer of MARV/Watsa titrated on the SW-13 cells was 3.41×10^6 $TCID_{50}$/ml and was neutralised by the bat serum to a titer of 7.34×10^5 $TCID_{50}$/ml. These results show that the bat serum was somewhat better able to neutralise the MARV/Ozolin strain, although the difference in neutralisation was not statistically significant (two-tailed p value: 0.47).

3.4 Discussion

This study reports on the duration of maternal immunity to MARV in both wild-caught and captive-born juvenile ERBs. In wild-caught juveniles, maternal IgG antibodies were present in all bats sampled at approximately 2 months of age, and were undetectable in all but one bat by 5 months of age. Likewise, in captive-born juveniles, maternal antibodies to MARV were undetectable in all bats by 5 months of age. These results show that maternal immunity is likely lost in juveniles between 4 and 5 months of age, making them susceptible to infection with MARV from 5 months of age onwards. These findings are consistent with previous estimates of the duration of maternal immunity to MARV (Paweska *et al*., 2015) and are in support of mathematical models and ecological studies of MARV infection in ERBs, which have suggested that the waning of maternal immunity in juvenile bats may be a major driver of MARV transmission and maintenance in nature (Amman *et al*., 2012; Peel *et al*., 2014; Hayman, 2015; Paweska *et al*., 2018). A longitudinal study by Amman and colleagues (2012) showed a peak in MARV infection rates in ERBs of around 6 months of age (MARV RNA detected in 12.4% of 6 month old juvenile ERBs compared to 2.7% in 3 month old ERBs and

0% in pups). Another ecological study reported the presence of MARV RNA in three juvenile bats of at least 6 months of age, but not in younger juveniles or in adult bats (Paweska *et al*., 2018). These observations point to a protective role of maternal antibodies against MARV infection in juvenile bats. The loss of maternal immunity to MARV in juvenile ERBs therefore increases the overall susceptibility of a bat population to MARV infection and may be linked to an increased risk of viral shedding and spillover into the human population.

From the data obtained in this study, predictions may be made for the period of greatest risk of MARV infection whilst entering caves inhabited by ERBs. South African ERBs are monoestric, with mating occurring between the months of June and September (Jacobsen & du Plessis, 1976; Penzhorn & Rautenbach, 1988; Paweska *et al*., 2018). After a gestational period of approximately 4 months (Kwiecinski & Griffiths, 1999), bats give birth to a single pup, with the majority of births occurring between the months of November and January (Paweska *et al*., 2018). Pups are weaned and start flying between 6 to 10 weeks of age (Kwiecinski & Griffiths, 1999), corresponding to the age of the bats when first sampled in this study. With maternal immunity lasting between 4 and 5 months, the majority of South African juvenile ERBs would become susceptible to MARV infection between the months of April and July. In support of these results, a study by Paweska and colleagues (2018) has indicated that the lowest seroprevalence for MARV (1.3%) in juvenile ERBs in a South African cave occurred during the month of June. Breeding patterns may differ between ERBs in different geographic locations due to environmental factors such as the availability of food, rainfall, temperature and daylight (Kwiecinski & Griffiths, 1999; Lucan *et al*., 2014), with biannual birthing seasons occurring in some ERB populations nearing the equator (Amman *et al*., 2012; Lucan *et al*., 2014). The periods of juvenile susceptibility and subsequent risk of spillover would therefore shift accordingly.

The duration of the antibody response of experimentally infected ERBs to MARV has previously been investigated by Schuh and colleagues (2017a). Their results showed a peak in anti-MARV IgG antibodies at a mean of 20 days p.i. (range: 14–28 days p.i.), corresponding to the period when viral shedding becomes undetectable (Schuh *et al*., 2017a). Immunoglobulin G antibodies to MARV then rapidly diminished and became undetectable within 3 months p.i. in both experimentally MARV-infected ERBs, and contact ERBs that had been "naturally"

infected by experimentally infected ERBs (Schuh *et al*., 2017a). In comparison, results from the current study as well as from a previous study (Paweska *et al*., 2015) showed a peak in anti-MARV IgG antibody levels in experimentally infected bats at day 14 p.i. (range: 9-21 days p.i.), with antibodies still being detectable in the majority of the bats (67%) almost 4 months after infection in the current study. There was considerable variation in the immune responses of each bat in this study, with some bats mounting a much greater immune response to MARV than others. The immunological responses of individual bats may be affected by age, past infections and breeding status (Plowright *et al*., 2008; Breed *et al*., 2011; Baker *et al*., 2014), although no clear distinction could be made between the immune responses of younger and older bats, or males and females in this study. It is possible that some bats possess natural genetic differences that allow them to retain longer lasting immunity to MARV compared to others, which may also explain why some bats in our study rapidly lost immunity to MARV, while others did not.

In contrast to results obtained from experimental inoculation studies (Schuh *et al*., 2017a; this study), results in bats naturally exposed to MARV suggest that anti-MARV IgG can persist in the majority of bats at moderately high levels for longer than 11 months. However, because the exposure histories of these bats to MARV are unknown, it is possible that the longer lasting IgG immune response may have resulted from re-exposure to the virus after primary infection had already occurred. The natural inoculation dose and ports of entry and exit of MARV in ERBs remain unclear. It was shown that bats may shed MARV in and possibly become infected through exposure to oral and fecal secretions (Amman *et al*., 2015). Infection in this manner may result in different immune responses in ERBs than infection through sub-cutaneous inoculation, which was the inoculation route chosen for the experimental infection studies discussed in this chapter (Paweska *et al*., 2015; Schuh *et al*., 2017a). Given the difference in results from studies of antibody-mediated immunity in experimentally and naturally infected ERBs, and possible differing routes and doses of experimental and natural MARV exposure, it is possible that results obtained in experimental infection studies might not accurately reflect what occurs upon natural exposure to MARV.

Further to evaluating the duration of the immune responses of ERBs to MARV, this study has indicated that bats harbouring antibodies to MARV can become reinfected upon re-exposure

with the virus, albeit with a heterologous isolate. The majority of reinfected bats in this study showed evidence of MARV in the blood, but virus presence in organs was mostly localised to the liver and spleen. This is in contrast to the systemic MARV replication observed in bats with a primary infection (Paweska *et al*., 2015). The presence of viraemia in seropositive bats after rechallenge, and the isolation of MARV from the serum of one bat on day 3 p.i. indicates the possibility of transmission of MARV via the blood for a short period of time following reinfection. This may occur through either haematophagous arthropods, or direct contact with infectious blood.

Replicating virus could not be isolated from any of the qRT-PCR positive tissues tested in this study. Unsuccessful attempts at isolating replicating virus from qRT-PCR positive tissues could possibly be ascribed to the viral load being below the detectable level, inadequate sensitivity of the virus isolation method used, or the presence of immune complexes. It has been shown that Ebola virus could not be isolated in Vero E6 culture from patient serum samples yielding cycle threshold (Ct) values higher than 33.7 (Jansen van Vuren *et al*., 2016), and in an ecological study of MARV infection in ERBs in Uganda, MARV could not be isolated in Vero cells from bat tissues with Ct values higher than 30 (Amman *et al*., 2012).

In this study, MARV could not be detected in major tissues that might play a role in viral shedding and transmission in bat populations, such as the salivary glands, intestine, reproductive tract and bladder. For this reason, it appears that reinfection of previously exposed bats might not play a major role in the maintenance of MARV in natural bat populations. However, different routes of infection might affect viral replication dynamics and tissue tropism. Physiological or environmental stress factors such as pregnancy, social stress or poor nutrition may also result in enhanced viral replication and shedding when reinfection takes place under such conditions (Marsh *et al*., 2012). It has been suggested that modified immune function during pregnancy may cause a temporary increase in the replication and shedding of filoviruses in African fruit bats (Pourrut *et al*., 2007). In addition, previous studies have provided evidence for increased horizontal viral transmission during pregnancy and lactation periods, as well as periods of food scarcity (Plowright *et al.*, 2008; Drexler *et al*., 2012; Baker *et al*., 2014). Schuh and colleagues (2017a) showed noticeable heterogeneities in the transmission of MARV between bats, with a small percentage of MARV-infected bats being

responsible for the majority of viral shedding (supershedders). Results from the current study showed a slight negative correlation between the robustness of the immune response and detectable viraemia in ERBs. It is therefore conceivable that, in a large ERB population, MARV reinfection may indeed lead to viral shedding in a number of bats that fail to mount a robust humoral immune response, and reinfection of these ERBs under stressful conditions may increase their probability of becoming supershedders. Furthermore, given the difference in sample size between this study (n = 15) and the previous study by Paweska and colleagues (2015) (n = 22), it is possible that MARV RNA might have been detected in some of these tissues if a larger number of bats had been included in the current study.

The mechanism by which MARV is able to infect bats that have pre-existing immunity to the virus needs further elucidation. Results of a previous study showed that bats with laboratory-induced immunity were able to efficiently control replication of the virus after re-exposure (Paweska *et al*., 2015). Similarly, Schuh and colleagues (2017b) were unable to demonstrate viraemia or the presence of MARV RNA in tissues sampled from MARV-reinfected bats with an apparent loss of immunity to the virus. The differences in the results obtained from work by Paweska and colleagues (2015) and the current study could be attributed to the timing of challenge and the status of immunity of the ERBs during re-exposure. Unlike the study conducted by Schuh and colleagues (2017b) in which bats had undetectable levels of antibodies to MARV prior to experimental reinfection, the bats used in the current study were collected with pre-existing natural immunity to MARV, without knowledge of the period that elapsed between the initial infection and reinfection in the laboratory. In addition, the rechallenge administered in both of the previous studies (Paweska *et al*., 2015; Schuh *et al*., 2017b) made use of homologous virus, while the virus used in the current study was heterologous. The challenge virus used in the current study originated from a human patient from the Democratic Republic of the Congo (DRC), whereas the virus circulating in the cave in Matlapitsi Valley is genetically distinct from the DRC isolate, and closely related to the MARV/Ozolin isolate (Paweska *et al*., 2018). Several genetically distinct strains and variants of MARV may circulate within a single cave system (Swanepoel *et al*., 2007; Towner *et al*., 2007; Towner *et al*., 2009; Amman *et al*., 2012), and the migratory nature of ERBs may result in the exchange of new strains of MARV between different colonies (Towner *et al*., 2009). It is possible that antibodies to one variant of MARV do not effectively neutralise variants that are genetically different. In

our evaluation of the capability of bat serum positive for anti-MARV/Matlapitsi antibodies to neutralise MARV/Watsa, it was shown that the antibodies were better able to neutralise an isolate closely related to the Matlapitsi variant (MARV/Ozolin) than the Watsa isolate. However, the difference in the reduction of the virus titers was not statistically significant. Whether immunity to one genetic variant of MARV is fully cross-protective against infection with another in ERBs should be explored further.

This study has not addressed the other aspects of immunity that might be important in the control of MARV in ERBs. The T cell responses produced following natural or experimental infection with MARV in ERBs remain to be evaluated. Antibody production does not necessarily correlate with viral clearance in bats (Middleton *et al*., 2007; Halpin *et al*., 2011). In addition, neutralising antibodies do not always confer protection *in vivo*, while-non-neutralising antibodies may confer protection through mechanisms such as antibody-dependent cell-mediated cytotoxicity. The correlates of protection against MARV in ERBs need to be determined.

In conclusion, the results from this study show that passive immunity to MARV is lost in juvenile ERBs between 4 and 5 months of age, making them susceptible to infection with the virus and increasing the risk for spillover into the human population when these bats first become infected with MARV. Exposure to MARV resulted in IgG immune responses lasting at least 110 days p.i. in the majority of experimentally infected bats, and at least 11 months in the majority of naturally infected bats. The results further suggest that antibodies to MARV in ERBs is likely not completely protective against reinfection. Future research should determine whether a complete loss of IgG antibodies to MARV may again lead to systemic infection and viral shedding in bats inoculated with different MARV isolates and subjected to different stress factors.

CHAPTER 4: FUTURE PERSPECTIVES AND CONCLUDING REMARKS

The continued expansion of the human population has led to the disruption of animal habitats, which has resulted in a risk for more frequent contact between humans and reservoir hosts for Marburg virus (MARV), and in turn, an increased risk for viral spillover and disease outbreaks. This increased risk necessitates enhanced surveillance for MARV in reservoir host populations and demands the development of safe and reliable diagnostic and surveillance tools. The current scarcity of data on the immune dynamics of Egyptian rousette bats (ERBs) to MARV has made it difficult to predict when and where outbreaks of disease may occur. While substantial progress has been made in regards to our understanding of the reservoir host-MARV relationship, much remains to be discovered. Understanding the antibody responses and dynamics of ERBs to MARV infection may assist in predicting when the risk of spillover to humans is greatest and consequently, in the development of strategies to reduce the risk of future spillover events. To this end, this study has aimed to provide tools for surveillance for MARV in bat populations, and to decipher the antibody responses of ERBs to MARV infection.

In chapter 2 of this thesis, the development and evaluation of two indirect enzyme-linked immunosorbent assays (I-ELISAs) based on the recombinant nucleoprotein (NP) and glycoprotein (GP) of MARV was described. Both I-ELISAs were found to be robust, highly sensitive and specific, and suitable for the detection of immunoglobulin G (IgG) antibodies to specific MARV proteins in ERB serum. Furthermore, the I-ELISAs are safe and practical tools for the monitoring and characterisation of the humoral immune responses of reservoir host bats to MARV.

By applying the serological tools developed in this study, some crucial aspects related to the humoral immune responses of reservoir host ERBs to MARV infection were addressed. These include the duration of maternal immunity to MARV in juvenile bats, the duration of humoral immunity generated upon active MARV infection in ERBs, the immune profile and dynamics of ERBs to two major MARV proteins, and the role of antibodies in the protection of ERBs against MARV reinfection. It was shown that maternal IgG antibodies are detectable in juvenile ERBs by an I-ELISA developed in this study, and that maternal immunity lasts between 4 and 5 months. If maternal immunity is protective against MARV infection, the loss of maternal

immunity in juvenile ERBs will significantly reduce the herd immunity of a bat population and increase the risk of MARV transmission and spillover into surrounding animal or human populations. The results of this study provide important information on the duration of maternal immunity to MARV in juvenile ERBs from which periods of potential spillover may be estimated, and MARV risk reduction strategies may be developed. Furthermore, the better understanding of the duration of maternal antibodies to MARV in ERBs will assist in determining whether anti-MARV IgG in young bats is maternally-derived or is the result of exposure to the virus. This study therefore also provides information that will allow for improved interpretation of serological data for MARV obtained from wild-caught ERBs. Maternal IgG antibodies are indistinguishable from actively obtained IgG antibodies by ELISA. The development of an ELISA for the detection of IgM to MARV in bats would therefore be important in order to accurately distinguish between maternally-derived antibodies and antibodies acquired by recent active infection. Unfortunately, few reagents and methodologies have been developed that would enable the complete characterisation of the immune repertoire of bats, and it is crucial that research in this regard continues.

In chapter 3, it was shown that ERBs produce long-term humoral immunity to MARV following experimental infection. Antibody responses to MARV GP peaked at 12 days p.i. and remained detectable in the majority of bats at the end of the study (110 days p.i.). The difference in the results obtained for the duration of humoral immunity to MARV in this study and a study by Schuh and colleagues (2017a) highlights the need for validated and standardised serological tests to compare results between different research laboratories effectively. The tools developed in this study have been evaluated for use with bat serum and will facilitate further research on the functional relevance of the antibody repertoire of ERBs in response to MARV infection.

Understanding how neutralising antibodies to MARV develop naturally in ERBs may provide important information regarding the mechanisms by which ERBs are able to control MVD, and may offer guidance for the design of potential vaccines. While this study has provided important new information regarding the ability of ERBs to produce long-lasting antibodies to the MARV GP, it is crucial that methods be developed that can detect and characterise MARV-neutralising antibody responses.

In this study, pre-existing immunity to MARV did not completely protect ERBs against MARV infection and replication; however, dissemination of the virus was limited, and shedding was only noted in one bat. Nevertheless, it is possible that reinfection of ERBs with MARV under stressful conditions such as starvation and pregnancy may lead to increased shedding in some individuals. Future research should focus on whether antibodies to MARV are cross-protective across different strains of the virus, and whether increased viral shedding will occur in response to different stress factors. It also remains to be determined whether ERBs may host persistent MARV infections. It is probable that both humoral and cellular immunity play important roles in the control of MARV infection in bats. The T cell responses of bats to MARV infection are poorly studied, as no reagents to quantify and identify T cell populations in bats have been developed. Development of such bat-specific reagents would greatly assist in elucidating the role of T cell responses in the protection of ERBs against MARV infection.

In conclusion, the immune status of a reservoir host population against specific MARV strains can influence several biological processes, including the emergence of new virus strains and the extent and severity of spillover events. Studying the immune responses to MARV in the natural reservoir host and comparing them to immune responses in humans and non-human primates may assist in identifying important processes involved in MARV disease susceptibility and transmission. Furthermore, continued study of bat immunology and immune dynamics will assist in understanding the ecology of MARV, developing strategies to prevent outbreaks in surrounding human and animal populations and improve preparedness for future epidemics. This study has provided important tools for surveillance and the characterisation of immune responses to MARV infection in ERB populations. In addition, this study has provided clues in regards to how antibody responses may play a role in the maintenance and transmission of MARV in bat populations. As MARV poses an ongoing threat to public health in Africa, further studies are essential to determine the major modes of transmission of the virus between bats, and to determine how this virus is able to persist in reservoir host bat colonies.

REFERENCES

Abbas AK, Lichtman AH and Pillai S (2014). Cellular and molecular immunity, 8th edition. Saunders: New York, pp 1-87.

Abraham C (2014). Break Ebola's stranglehold. *New Scientist* **223**: 22-23.

African Chiroptera Report (2017). African Chiroptera Report. Van Cakenberghe V and Seamark ECJ, editors. African Bats: Pretoria, pp 129-145.

Adjemian J, Farnon EC, Tschioko F, Wamala JF, Byaruhanga E, Bwire GS, Kansiime E, Kagirita A, Ahimbisibwe S, Katunguka, Jeffs B, Lutwama JJ, Downing R, Tappero JW, Formenty P, Amman B, Manning C, Towner J, Nichol ST and Rollin PE. (2011). Outbreak of Marburg hemorrhagic fever among miners in Kamwenge and Ibanda Districts, Uganda, 2007. *J Infect Dis* **204**: S796-S799.

Adu-Gyamfi E, Soni SP, Jee CS, Digman MA, Gratton E and Stahelin RV (2014). A loop region in the N-terminal domain of Ebola virus VP40 is important in viral assembly, budding, and egress. *Viruses* **6**: 3837-3854.

Agrati C, Castilletti C, Casetti R, Sacchi A, Falasca L, Turchi F, Tumino N, Bordoni V, Cimini E, Viola D, Lalle E, Bordi L, Lanini S, Martini F, Nicastri E, Petrosillo N, Puro V, Piacentini M, Di Caro A, Kobinger GP, Zumla A, Ippolito G and Capobianchi MR (2016). Longitudinal characterization of dysfunctional T cell-activation during human acute Ebola infection. *Cell Death Dis* **7**: e2164.

Ahn M, Cui J, Irving AT and Wang L-F (2016). Unique loss of the PYHIN gene family in bats amongst mammals: implications for inflammasome sensing. *Sci Rep* **6**: 21722.

Amarasinghe GK, Ceballos NGA, Banyard AC, Basler CF, Bavari S, Bennett AJ, Blasdell KR, Briese T, Bukreyev A, Cai Y, Calisher CH, Lawson CC, Chandran K, Chapman CA, Chiu CY, Choi K-S, Collins PL, Dietzgen RG, Dolja VV, Dolnik O, Domier LL, Durrwald R, Dye JM,

Easton AJ, Ebihara H, Echevarria JE, Fooks AR, Formenty PBH, Fouchier RAM, Freuling CM, Ghedin E, Goldberg TL, Hewson R, Horie M, Hyndman TH, Jiang D, Kityo R, Kobinger GP, Kondo H, Koonin EV, Krupovic M, Kurath G, Lamb RA, Lee B, Leroy EM, Maes P, Maisner A, Marston DA, Mor SK, Müller T, Mühlberger E, Ramírez VMN, Netesov SV, Ng TFF, Nowotny N, Palacios G, Patterson JL, Paweska JT, Payne SL, Prieto K, Rima BK, Rota P, Rubbenstroth D, Schwemmle M, Siddell S, Smither SJ, Song Q, Song T, Stenglein MD, Stone DM, Takada A, Tesh RB, Thomazelli LM, Tomonaga K, Tordo N, Towner JS, Vasilakis N, Vázquez-Morón S, Verdugo C, Volchkov VE, Wahl V, Walker PJ, Wang D, Wang L-F, Wellehan JFX, Wiley MR, Whitfield AE, Wolf YI, Yè G, Zang Y-Z and Kuhn JH (2016). Taxonomy of the order *Mononegavirales*: update 2018. *Arch Virol* doi: 10.1007/s00705-018-3814-x (E-pub ahead of print).

Ali MT and Islam MO (2015). A highly conserved GEQYQQLR epitope has been identified in the nucleoprotein of Ebola virus by using an in silico approach. *Adv Bioinformatics* **2015**: 278197.

Allen LC, Turmelle AS, Mendonca MT, Navara KJ, Kunz TH and McCracken GF (2008). Roosting ecology and variation in adaptive and innate immune system function in the Brazilian free-tailed bat (*Tadarida brasiliensis*). *J Comp Physiol B* **179**: 315-323.

Alves DA, Glynn AR, Steele KE, Lackemeyer MG, Garza NL, Buck JG, Mech C and Reed DS (2010). Aerosol exposure to the Angola strain of Marburg virus causes lethal viral hemorrhagic fever in cynomolgus macaques. *Vet Pathol* **47**: 831-851.

Amman BR, Carroll SA, Reed ZD, Sealy TK, Balinandi S, Swanepoel R, Kemp A, Erickson BR, Comer JA, Campbell S, Cannon DL, Khristova ML, Atimnedi P, Paddock CD, Kent Crockett RJ, Flietstra TD, Warfield KL, Unfer R, Katongole-Mbidde E, Downing R, Tappero JW, Zaki SR, Rollin PE, Ksiazek TG, Nichol ST and Towner JS (2012). Seasonal pulses of Marburg virus circulation in juvenile *Rousettus aegyptiacus* bats coincide with periods of increased risk of human infection. *PLoS Pathog* **8**: e1002877.

Amman BR, Jones MEB, Sealy TK, Uebelhoer LS, Schuh AJ, Bird BH, Coleman-McCray JD, Martin BE, Nichol ST and Towner JS (2015). Oral shedding of Marburg virus in experimentally infected Egyptian fruit bats (*Rousettus aegyptiacus*). *J Wildlife Dis* **51**: 113-124.

Audet J and Kobinger GP (2015). Immune evasion in Ebolavirus infections. *Viral Immunol* **28**: 1-9.

Ayouba A, Touré A, Butel C, Keita AK, Binetruy F, Sow MS, Foulongne V, Delaporte E and Peeters M (2017). Development of a sensitive and specific serological assay based on Luminex technology for detection of antibodies to Zaire Ebola virus. *J Clin Microbiol* **55**: 165-176.

Baize S, Leroy EM, Georges-Courbot MC, Capron M, Lansoud-Soukate J, Debré P, Fisher-Hoch SP, McCormick JB and Georges AJ (1999). Defective humoral responses and extensive intravascular apoptosis are associated with fatal outcome in Ebola virus-infected patients. *Nat Med* **5**: 423-426.

Baize S, Leroy EM, Georges AJ, Georges-Courbot MC, Capron M, Bedjabaga I, Lansoud-Soukate J and Mavoungou E (2002). Inflammatory responses in Ebola virus-infected patients. *Clin Exp Immunol* **128**: 163-168.

Baize S, Pannetier D, Oestereich L, Rieger T, Koivogui L, Magassouba N, Soropogui B, Sow MS, Keïta S, De Clerck H, Tiffany A, Dominguez G, Loua M, Traoré A, Kolié M, Malano ER, Heleze E, Bocquin A, Mély S, Raoul H, Caro V, Cadar D, Gabriel M, Pahlmann M, Tappe D, Schmidt-Chanasit J, Impouma B, Diallo AK, Formenty P, Van Herp M and Günther S (2014). Emergence of Zaire Ebola virus disease in Guinea. *N Engl J Med* **371**: 1418-1425.

Baker ML, Tachedjian M and Wang LF (2010). Immunoglobulin heavy chain diversity in Pteropid bats: evidence for a diverse and highly specific antigen binding repertoire. *Immunogenetics* **62**: 173-184.

Baker ML, Schountz T and Wang L-F (2013). Antiviral immune responses of bats: a review. *Zoonoses Public Health* **60**: 104-116.

Baker KS, Suu-Ire R, Barr J, Hayman DTS, Broder CC, Horton DL, Durrant C, Murcia PR, Cunningham AA and Wood JLN (2014). Viral antibody dynamics in a chiropteran host. *J Animal Ecol* **83**: 451-428.

Baker ML and Zhou P (2015). Bat immunology. In: Wang L-F and Cowled C, editors. Bats and viruses: a new frontier of emerging infectious diseases. Wiley & Sons: New Jersey, pp 327-342.

Baker LE, Ellena JF, Handing KB, Derewenda U, Utepbergenov D, Engel DA and Derewenda ZS (2016). Molecular architecture of the nucleoprotein C-terminal domain from the Ebola and Marburg viruses. *Acta Crystallogr D Struct Biol* **72**: 49-58.

Bale S, Julien JP, Bornholdt ZA, Kimberlin CR, Halfmann P, Zandonatti MA, Kunert J, Kroon GJ, Kawaoka Y, MacRae IJ, Wilson IA and Saphire EO (2012a). Marburg virus VP35 can both fully coat the backbone and cap the ends of dsRNA for interferon antagonism. *PLoS Pathog* **8**: e1002916.

Bale S, Dias JM, Fusco ML, Hashiguchi T, Wong AC, Liu T, Keuhne AI, Li S, Woods VL Jr, Chandran K, Dye JM and Ollman Saphire E (2012b). Structural basis for differential neutralization of ebolaviruses. *Viruses* **4**: 447-470.

Bamberg S, Kolesnikova L, Möller P, Klenk HD and Becker S (2005). VP24 of Marburg virus influences formation of infectious particles. *J Virol* **79**: 13421-13433.

Banerjee A, Misra V, Schountz T and Baker ML (2018). Tools to study pathogen-host interactions in bats. *Virus Res* **248**: 5-12.

Barclay RMR and Jacobs DS (2011). Differences in the foraging behaviour of male and female Egyptian fruit bats (*Rousettus aegyptiacus*). *Can J Zool* **89**: 466-473.

Bausch DG, Nichol ST, Muyembe-Tamfum JJ, Borchert M, Rollin PE, Sleurs H, Campbell P, Tshioko FK, Roth C, Colebunders R, Pirard P, Mardel S, Olinda LA, Zeller H, Tshomba A, Kulidri A, Libande ML, Mulangu S, Formenty P, Grein T, Leirs H, Braack L, Ksiazek T, Zaki S, Bowen MD, Smit SB, Leman PA, Burt FJ, Kemp A and Swanepoel R (2006). Marburg hemorrhagic fever associated with multiple genetic lineages of virus. *N Engl J Med* **355**: 909-919.

Bean AGD, Maker ML, Stewart CR, Cowled C, Deffrasnes C, Wang L-F and Lowenthal JW (2013). Studying immunity to zoonotic diseases in the natural host – keeping it real. *Nat Rev Immunol* **13**: 851-861.

Becquart P, Mahlakoiv T, Nkoghe D and Leroy EM (2014). Identification of continuous human B-cell epitopes in the VP35, VP40, nucleoprotein and glycoprotein of Ebola virus. *PLoS ONE* **9**: e96360.

Bermejo M, Rodriguez-Teijeiro JD, Illera G, Barroso A, Vila C and Walsh PD (2006). Ebola outbreak killed 5 000 gorillas. *Science* **314**: 1564.

Bhak Y, Jeon Y, Jeon S, Chung O, Jho S, Jun J, Kim H-K, Cho Y, Yoon C, Lee S, Kang J-H, Lim J-D, An J, Cho YS, Ryu D-Y and Bhak J (2017). *Myotis rufoniger* genome sequence and analyses: *M. rufoniger*'s genomic feature and the decreasing effective population size of *Myotis* bats. *PLoS ONE* **12**: e0180418.

Bharat TA, Riches JD, Kolesnikova L, Welsch S, Krahling V, Davey N, Parsy ML, Becker S and Briggs JA (2011). Cryo-electron tomography of Marburg virus particles and their morphogenesis within infected cells. *PLoS Biol* **9**: e1001196.

Biedenkopf N, Schlereth J, Grunweller A, Becker S and Hartmann RK (2016). RNA-binding of Ebola virus VP30 is essential for activating viral transcription. *J Virol* **90**: 7481-7496.

Bosio CM, Aman MJ, Grogan C, Hogan R, Ruthel G, Negley D, Mohamadzadeh M, Bavari S and Schmaljohn A (2003). Ebola and Marburg viruses replicate in monocyte-derived dendritic

cells without inducing the production of cytokines and full maturation. *J Infect Dis* **188**: 1630-1638.

Bower H and Glyn JR (2017). A systematic review and meta-analysis of seroprevalence surveys of ebolavirus infection. *Scientific Data* **4**: 160133.

Brauburger K, Hume AJ, Mühlberger E and Olejnik J (2012). Forty-five years of Marburg virus research. *Viruses* **4**: 1878-1927.

Bray M (2001). The role of the type I interferon response in the resistance of mice to filovirus infection. *J Gen Virol* **82**:1365-1373.

Breed AC, Breed MF, Meers J and Field HE (2011). Evidence of endemic Hendra virus infection in flying foxes (*Pteropus conspicillatus*) - implications for disease risk management. *PLoS ONE* **6**: e28816.

Broadhurst MJ, Brooks TJG and Pollock NR (2016). Diagnosis of Ebola virus disease: past, present and future. *Clin Microbiol Rev* **29**: 773-793.

Brunet-Rossini AK and Wilkinson GS (2009). Methods for age estimation and the study of senescence in bats. In: Kuhn TH and Parsons S, editors. Ecological and behavioral methods for the study of bats. The John Hopkins University Press: Baltimore, pp 315-325.

Bukreyev AA, Volchkov VE, Blinov VM, Dryga SA and Netesov SV (1995). The complete nucleotide sequence of the Popp (1967) strain of Marburg virus: a comparison with the Musoke (1980) strain. *Arch Virol* **140**: 1589-1600.

Butler JE, Wertz N, Zhao Y, Zhang S, Bao Y, Bratsch S, Kunz TH, Whitaker JO Jr and Schountz T (2011). The two suborders of chiropterans have the canonical heavy chain immunoglobulin (Ig) gene repertoire of eutherian mammals. *Dev Comp Immunol* **35**: 273-284.

Calisher CH, Childs JE, Field HE, Holmes KV and Schountz T (2006). Bats: important reservoir hosts of emerging viruses. *Clinical Microbiology Reviews* **19**: 531-545.

Callendret B, Vellinga J, Wunderlich K, Rodriguez A, Steigerwald R, Dirmeier U, Cheminay C, Volkmann A, Brasel T, Carrion R, Giavedoni LD, Patterson JL, Mire CE, Geisbert TW, Hooper JW, Weijtens M, Hartkoorn-Pasma J, Custers J, Grazia Pau M, Schuitemaker H and Zahn R (2018). A prophylactic multivalent vaccine against different filovirus species is immunogenic and provides protection from lethal infections with Ebolavirus and Marburgvirus species in non-human primates. *PLoS ONE* **13**: e0192312.

Carrion R Jr, Ro Y, Hoosien K, Ticer A, Brasky K, de la Garza M, Mansfield K and Patterson JL (2011). A small non-human primate model for filovirus-induced disease. *Virology* **420**: 117-124.

Carroll SA, Towner JS, Sealy TK, McMullan LK, Khristova ML, Burt FJ, Swanepoel R, Rollin PE and Nichol ST (2013). Molecular evolution of viruses of the family *Filoviridae* based on 97 whole genome sequences. *J Virol* **87**: 2608-2616.

Centres for Disease Control and Prevention (2009). Imported case of Marburg hemorrhagic fever - Colorado, 2008. *MMWR Morb Mortal Wkly Rep* **58**: 1377-1381.

Chakraborty AK and Chakravarty AK (1984). Antibody mediated immune response in the bat, *Pteropus giganteus*. *Dev Comp Immunol* **8**: 415–423.

Chakravarty A and Sarkar S (1994). Immunofluorescence analysis of immunoglobulin bearing lymphocytes in the Indian fruit bat: *Pteropus giganteus*. *Lymphology* **27**: 97-104.

Changula K, Yoshida R, Noyori O, Marzi A, Miyamoto H, Ishijima M, Yokoyama A, Kajihara M, Feldmann H, Mweene AS and Takada A (2013). Mapping of conserved and species-specific antibody epitopes on the Ebola virus nucleoprotein. *Virus Res* **176**: 83-90.

Cliquet F and Sagne MAL (1998). Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. *J Immunol Methods* **212**: 79-87.

Cogswell-Hawkinson A, Bowen R, James S, Gardiner D, Calisher CH, Adams R and Schountz T (2012). Tacaribe virus causes fatal infection of an ostensible reservoir host, the Jamaican fruit bat. *J Virol* **86**: 5791-5799.

Connor JH, Yen J, Caballero IS, Garamszegi S, Malhotra S, Lin K, Hensley L and Goff AJ (2015). Transcriptional profiling of the immune response to Marburg virus infection. *J Virol* **89**: 9865-9874.

Conrad JL, Isaacson M, Smith EB, Wulff H, Crees M, Geldenhuys P and Johnston J (1978). Epidemiologic investigation of Marburg virus disease, Southern Africa, 1975. *Am J Trop Med Hyg* **27**: 1210-1215.

Cook JD and Lee JE (2013). The secret life of viral entry glycoproteins: moonlighting in immune evasion. *PLoS Pathog* **9**: e1003258.

Cote M, Misai J, Ren T, Bruchez A, Lee K, Filone CM, Hensley L, Li Q, Ory D, Chandran K and Cunningham J (2011). Small molecule inhibitors reveal Niemann-Pick C1 is essential for Ebola virus infection. *Nature* **477**: 344-348.

Cowled C, Baker M, Tachedjian M, Zhou P, Bulach D and Wang L-F (2011). Molecular characterisation of toll-like receptors in the black flying fox *Pteropus alecto*. *Dev Comp Immunol* **36**: 657-664.

Cross RW, Fenton KA, Geisbert JB, Ebihara H, Mire CE and Geisbert TW (2015). Comparison of the pathogenesis of the Angola and Ravn strains of Marburg virus in the outbred guinea pig model. *J Infect Dis* **212**: S258-S270.

Crowther JR (2009). The ELISA guidebook. In: Methods in Molecular Biology, 2nd edition, volume 516. Humana Press: New Jersey, pp 79-334.

Dolnik O, Kolesnikova L, Stevermann L and Becker S (2010). Tsg101 is recruited by a late domain of the nucleocapsid protein to support budding of Marburg virus-like particles. *J Virol* **84**: 7847-7856.

Dowling W, Thompson E, Badger C, Mellquist JL, Garrison AR, Smith JM, Paragas J, Hogan RJ and Schmaljohn C (2007). Influences of glycosylation on antigenicity, immunogenicity, and protective efficacy of Ebola virus GP DNA vaccines. *J Virol* **81**:1821-1837.

Drexler JF, Corman VM, Gloza-Rausch F, Muller MA and Drosten C (2012). Amplification of emerging RNA viruses associated with formation and parturition in a bat maternity roost. *EcoHealth* **000**: 223.

Dupuis S, Jouanguy E, Al-Hajjar S, Fieschi C, Al-Mohsen IZ, Al-Jumaah S, Yang K, Chapgier A, Eidenschenk C, Eid P, Al Ghonaium A, Tufenkeji H, Frayha H, Al-Gazlan S, Al-Rayes H, Schreiber RD, Gresser I and Casanova JL (2003). Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. *Nat Genet* **33**: 388-391.

Edmond RT, Evans B, Bowen ET and Lloyd G (1977). A case of Ebola virus infection. *Br Med J* **27**: 541-544.

Edwards MR, Johnson B, Mire CE, Xu W, Shabman RS, Speller LN, Leung DW, Geisbert TW, Amarasinghe GK and Basler CF (2014). The Marburg virus VP24 protein interacts with Keap1 to activate the cytoprotective antioxidant response pathway. *Cell Rep* **6**: 1017-1025.

Enterlein S, Volchkov V, Weik M, Kolesnikova L, Volchkova V, Klenk HD and Mühlberger E (2006). Rescue of recombinant Marburg virus from cDNA is dependent on nucleocapsid protein VP30. *J Virol* **80**: 1038-1043.

Epstein JH, Baker ML, Zambrana-Torrelio C, Middleton D, Barr JA, DuBovi E, Boyd V, Pope B, Todd S, Crameri G, Walsh A, Pelican K, Fielder MD, Davies AJ, Wang L-F and Daszak P (2013). Duration of maternal antibodies against canine distemper virus and Hendra virus in pteropid bats. *PLoS ONE* **8**: e67584.

Feldmann H, Will C, Schikore M, Slenczka W and Klenk HD (1991). Glycosylation and oligomerization of the spike protein of Marburg virus. *Virology* **182**: 353-356.

Feldmann H and Klenk HD (1996). Filoviruses. In: Baron S, editor. Medical Microbiology, 4th edition. University of Texas Medical Branch: Texas.

Feldmann H, Sanchez A and Geisbert TW (2013). Filoviridae: Marburg and Ebola viruses. In: Knipe DM and Howley PM, editors. Fields virology, 6th edition. Lippincott Williams & Wilkins: Philadelphia, pp 923-956.

Fernando L, Qiu X, Melito PL, Williams KJN, Feldmann F, Feldmann H, Jones SM and Alimonti JB (2015). Immune responses to Marburg virus Angola infection in nonhuman primates. *J Infect Dis* **212**: S234-S241.

Flyak AI, Ilinykh PA, Murin CD, Garron T, Shen X, Fusco ML, Hashiguchi T, Bornholdt ZA, Slaughter JC, Sapparapu G, Klages C, Ksiazek TG, Ward AB, Ollmann Saphire E, Bukreyev A and Crowe JE Jr (2015). Mechanism of human antibody-mediated neutralization of Marburg virus. *Cell* **160**: 893-903.

Francica JR, Matukonis MK and Bates P (2009). Requirements for cell rounding and surface protein down-regulation by Ebola virus glycoprotein. *Virology* **383**: 237-247.

Francica JR, Varela-Rohena A, Medvec A, Plesa G, Riley JL and Bates P (2010). Steric shielding of surface epitopes and impaired immune recognition induced by the Ebola virus glycoprotein. *PLoS Pathog* **6**: e1001098.

Fritz EA, Geisbert JB, Geisbert TW, Hensley LE and Reed DS (2008). Cellular immune response to Marburg virus infection in cynomolgus macaques. *Viral Immunol* **21**: 355-363.

Fujii H, Watanabe S, Yamane D, Ueda N, Iha K, Taniguchi S, Kato K, Tohya Y, Kyuwa S, Yoshikawa Y and Akashi H (2010). Functional analysis of *Rousettus aegyptiacus* "signal transducer and activator of transcription 1" (STAT1). *Dev Comp Immunol* **34**: 598-602.

Fusco ML, Hashiguchi T, Cassan R, Biggins JE, Murin CD, Warfield KL, Li S, Holtsberg FW, Shulenin S, Vu H, Olinger GG, Kim DH, Whaley KJ, Zeitlin L, Ward AB, Nykiforuk C, Aman MJ, Berry J and Ollman Saphire E (2015). Protective mAbs and cross-reactive mAbs raised by immunisation with engineered Marburg virus GPs. *PLoS Pathog* **11**: e1005016.

Gear JS, Cassel GA, Gear AJ, Trappler B, Clausen L, Meyers AM, Kew MC, Bothwell TH, Sher R, Miller GB, Schneider J, Koornhof HJ, Gomperts ED, Isaacson M and Gear JH (1975). Outbreak of Marburg virus disease in Johannesburg. *Br Med J* **4**: 489-493.

Geisbert TW and Jahrling PB (1995). Differentiation of filoviruses by electron microscopy. *Virus Res* **39**: 129-150.

Geisbert TW, Pushko P, Anderson K, Smith J, Davis KJ and Jahrling PB (2002). Evaluation in nonhuman primates of vaccines against Ebola virus. *Emerg Infect Dis* **8**: 503-507.

Geisbert TW, Young HA, Jahrling PB, Davis KJ, Larsen T, Kagan E and Hensley LE (2003). Pathogenesis of Ebola haemorrhagic fever in primate models: evidence that haemorrhage is not a direct effect of virus-induced cytolysis of endothelial cells. *Am J Pathol* **163**: 2371-2382.

Geisbert TW, Daddario-DiCaprio KM, Geisbert JB, Young HA, Formenty P, Fritz EA, Larsen T and Hensley LE (2007). Marburg virus Angola infection of rhesus macaques: pathogenesis and treatment with recombinant nematode anticoagulant protein c2. *J Infect Dis* **196**: S372-S381.

Gerpe MCR, Renner TM, Belanger K, Lam C, Aydin H and Langlois M-A (2015). N-linked glycosylation protects gammaretroviruses against deamination by APOBEC3 proteins. *J Virol* **89**: 2342- 357.

Gonchar NI, Pshenichnov VA, Pokhodiaev VA, Lopatov KL and Firsova IV (1991). The sensitivity of different experimental animals to the Marburg virus. *Vopr Virusol* **36**: 435-437.

Gonzalez JP, Nakoune E, Slenczka W, Vidal P and Morvan JM (2000). Ebola and Marburg virus antibody prevalence in selected populations of the Central African Republic. *Microbes Infect* **2**: 39-44.

Groen J, van den Hoogen BG, Burghoorn-Maas CP, Fooks AR, Burton J, Clegg CJ, Zeller H and Osterhaus AD (2003). Serological reactivity of baculovirus-expressed Ebola virus VP35 and nucleoproteins. *Microbes Infect* **5**: 379-385.

Groseth A, Jones S, Artsob H and Feldmann H (2009). Hemorrhagic fever viruses as biological weapons. In: Fong IW and Alibeck K, editors. Bioterrorism and infectious agents: a new dilemma for the 21st century. Springer-Verlag: New York, pp 169-191.

Gupta M, Mahanty S, Greer P, Towner JS, Shieh WJ, Zaki SR, Ahmed R and Rollin PE (2004). Persistent infection with Ebola virus under conditions of partial immunity. *J Virol* **78**: 958-967.

Hall TA (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser* **41**: 95-98.

Halpin K, Hyatt AD, Fogarty R, Middleton D, Bingham J, Epstein JH, Rahman SA, Hughes T, Smith C, Field HE, Daszak P and the Henipavirus Ecology Research Group (2011). Pteropid bats are confirmed as the reservoir hosts of henipaviruses: a comprehensive experimental study of virus transmission. *Am J Trop Med Hyg* 2011 **85**: 946-951.

Hartman AL, Towner JS and Nichol ST (2004). A C-terminal basic amino acid motif of Zaire

ebolavirus VP35 is essential for type I interferon antagonism and displays high identity with the RNA-binding domain of another interferon antagonist, the NS1 protein of influenza A virus. *Virology* **328**: 177-184.

Hatten BA, Allen R and Sulkin SE (1968). Immune response in Chiroptera to bacteriophage phiX174. *J Immunol* **101**: 141-150.

Hayman DT (2015). Biannual birth pulses allow filoviruses to persist in bat populations. *Proc Biol Sci* **282**: 20142591.

He G, He B, Racey P and Cui J (2010). Positive selection of the bat interferon alpha gene family. *Biochem Genet* **48**: 840-846.

Hensley LE, Alves DA, Geisbert JB, Fritz EA, Reed C, Larsen T and Geisbert TW (2011). Pathogenesis of Marburg hemorrhagic fever in cynomolgus macaques. *J Infect Dis* **204**: S1021-S1031.

Hevey M, Negley D and Schmaljohn A (2003). Characterization of monoclonal antibodies to Marburg virus (strain Musoke) glycoprotein and identification of two protective epitopes. *Virology* **314**: 350-357.

Hölzer M, Krähling V, Amman F, Barth E, Bernhart SH, Carmelo VAO, Collatz M, Doose G, Eggenhofer F, Ewald J, Fallmann J, Feldhahn LM, Fricke M, Gebauer J, Gruber AJ, Hufsky F, Indrischek H, Kanton S, Linde J, Mostajo N, Ochsenreiter R, Riege K, Rivarola-Duarte L, Sahyoun AH, Saunders SJ, Seemann SE, Tanzer A, Vogel B, Wehner S, Wolfinger MT, Backofen R, Gorodkin J, Grosse I, Hofacker I, Hoffmann S, Kaleta C, Stadler PF, Becker S and Marz M (2016). Differential transcriptional responses to Ebola and Marburg virus infection in bat and human cells. *Sci Rep* **6**: 34589

Huang Y, Zhu Y, Yang M, Zhang Z, Song D and Yuan Z (2014). Nucleoprotein-based indirect enzyme-linked immunosorbent assay (indirect ELISA) for detecting antibodies specific to Ebola virus and Marburg virus. *Virol Sin* **29**: 372-380.

Hume AJ, Ames J, Rennick LJ, Duprex WP, Marzi A, Tonkiss J and Mühlberger E (2016). Inactivation of RNA viruses by gamma irradiation: a study on mitigating factors. *Viruses* **8**: 204.

Iha K, Omatsu T, Watanabe S, Ueda N, Taniguchi S, Fujii H, Ishii Y, Kyuwa S, Akashi H and Yoshikawa Y (2009). Molecular cloning and sequencing of the cDNAs encoding bat interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12p40, and tumor necrosis factor-alpha. *J Vet Med Sci* **71**: 1691-1695.

Iha K, Omatsu T, Watanabe S, Ueda N, Taniguchi S, Fujii H, Ishii Y, Kyuwa S, Akashi H and Yoshikawa Y (2010). Molecular cloning and expression analysis of bat toll-like receptors 3, 7 and 9. *J Vet Med Sci* **72**: 217-220.

Ikegami T, Saijo M, Niikura M, Miranda ME, Calaor AB, Hernandez M, Manalo DL, Kurane I, Yoshikawa Y and Morikawa S (2002). Development of an immunofluorescence method for the detection of antibodies to Ebola virus subtype Reston by the use of recombinant nucleoprotein-expressing HeLa cells. *Microbiol Immunol* **46**: 633-638.

Isaacson M, Sureau P, Courteille G and Pattyn SR (1978). Clinical aspects of Ebola virus disease at the Ngaliema Hospital, Kinshasa, Zaire. In: Pattyn SR, editor. Ebola virus haemorrhagic fever. Elsevier Academic Press: Amsterdam, pp 22-26.

Jacobsen NHG and du Plessis E (1976). Observations on the ecology and the biology of the Cape fruit bat *Roussetous aegyptiacus leachi* in Eastern Transvaal. *S Afr J Sci* **72**: 270-273.

Jacobsen RH (1998). Validation of serological assays for diagnosis of infectious diseases. *Rev Sci Tech Off Int Epiz* **17**: 469-486.

Jansen van Vuren P, Tiemessen CT and Paweska JT (2010). Evaluation of a recombinant Rift Valley fever virus subunit nucleocapsid protein as an immunogen in mice and sheep. *Open Vaccine Journal* **3**: 114-126.

Jansen van Vuren P, Grobbelaar A, Storm N, Conteh O, Konneh K, Kamara A, Sanne I and Paweska JT (2016). Comparative evaluation of the diagnostic performance of the prototype Cepheid GeneXpert Ebola assay. *J Clin Microbiol* **52**: 359-367.

Jarvis DL, Kawar ZS and Hollister JR **(**1998). Engineering N-glycosylation pathways in the baculovirus-insect cell system. *Curr Opin Biotechnol* **9**: 528-533.

Jeffers SA, Sanders DA and Sanchez A (2002). Covalent modifications of the Ebola virus glycoprotein. *J Virol* **76**: 12463-12472.

Johnson KM, Elliot LH and Heymann DL (1981). Preparation of polyvalent viral immunofluorescent intracellular antigens and use in human serosurveys. *J Clin Microbiol* **14**: 527-529.

Johnson ED, Johnson BK, Silverstein D, Tukei P, Geisbert TW, Sanchez AN and Jahrling PB (1996). Characterization of a new Marburg virus isolated from a 1987 fatal case in Kenya. *Arch Virol* **11**: 101-114.

Kajihara M and Takada A (2015). Host cell factors involved in filovirus infection. *Curr Trop Med Rep* **2**: 30-40.

Kaletsky RL, Francica JR, Agrawal-Gamse C and Bates P (2009). Tetherin-mediated restriction of filovirus budding is antagonized by the Ebola glycoprotein. *Proc Natl Acad Sci USA* **106**: 2886-2891.

Kamata T, Natesan M, Warfield K, Aman MJ and Ulrich RG (2014). Determination of specific antibody responses to the six species of Ebola and Marburg viruses by multiplexed protein microarrays. *Clin Vaccine Immunol* **21**: 1605-1612.

Kärber G (1931). Bitrag zur kollektiven behandlung pharmakologischer reihenversuche. *Naunyn-Schmiedeberg's Arch exp Path Pharmak* **162**: 480-487.

Karesh WB, Dobson A, Lloyd-Smith JO, Lubroth J, Dixon MA, BennettM, Aldrich S, Harrington T, Formenty P, Loh EH, Machalaba CC, Thomas MJ and Heymann DL (2012). Ecology of zoonoses: natural and unnatural histories. *Lancet* **380**: 1936-1945.

Kepler T, Sample C, Hudak K, Roach J, Haines A, Walsh A and Ramsburg E (2010). Chiropteran types I and II interferon genes inferred from genome sequencing traces by a statistical gene-family assembler. *BMC Genomics* **11**: 444.

Khalil HM, Abdel Aal TM, Maklad MKH, Abdallah HM, Fahmy IA and Elzayyat EA (1990). Specificity of crude and purified Fasciola antigens in immunodiagnosis of human fascioliasis. *J Egypt Soc Parasitol* **20**: 87-94.

Klasse PJ (2014). Neutralization of virus infectivity by antibodies: old problems in new perspectives. *Advances in Biology* **2014**: 1-24.

Knust B, Schafer IJ, Wamala J, Nyakarahuka L, Okot C, Shoemaker T, Dodd K, Gibbons A, Balinandi S, Tumusiime A, Campbell S, Newman E, Lasry E, DeClerck H, Boum Y, Makumbi I, Bosa HK, Mbonye A, Aceng JR, Nichol ST, Ströher U and Rollin PE (2015). Multidistrict outbreak of Marburg virus disease - Uganda, 2012. *J Infect Dis* **212**: S119-S128.

Kobinger GP, Leung A, Neufeld J, Richardson JS, Falzarano D, Smith G, Tierney K, Patel A and Weingartl HM (2011). Replication, pathogenicity, shedding and transmission of Zaire ebolavirus in pigs. *J Infect Dis* **204**: 200-208.

Kozak R, He S, Kroeker A, de la Vega M-A, Audet J, Wong G, Urfano C, Antonation K, Embury-Hyatt C, Kobinger GP and Qiu X (2016). Ferrets infected with Bundibugyo virus or Ebola virus recapitulate important aspects of human filovirus disease. *J Virol* **90**: 9209-9223.

Ksiazek TG, West CP, Rollin PE, Jahrling PB and Peters CJ (1999). ELISA for the detection of antibodies to filoviruses. *J Infect Dis* **179**: S192-S198.

Kühl A, Banning C, Marzi A, Votteler J, Steffen I, Bertram S, Glowacka I, Konrad A, Sturzl M, Guo JT, Schubert U, Feldmann H, Behrens G, Schindler M and Pohlmann S (2011). The Ebola virus glycoprotein and HIV-1 Vpu employ different strategies to counteract the antiviral factor tetherin. *J Infect Dis* **204**: S850-860.

Kuhn JH (2008). Filoviruses: A compendium of 40 years of epidemiological, clinical, and laboratory studies. *Arch Virol Suppl* **20**:13-360.

Kumar H, Taro K and Shizuo A (2011). Pathogen recognition by the innate immune system. *Int Rev Immunol* **30**: 16-34.

Kutalek R, Wang S, Fallah M, Wesseh CS and Gilbert J (2015). Ebola interventions: listen to communities. *Lancet Glob Health* **3**: e131.

Kuzmin IV, Niezgoda M, Franka R, Agwanda B, Markotter W, Breiman RF, Shieh WJ, Zaki SR and Rupprecht CE (2010). Marburg virus in fruit bat, Kenya. *Emerg Infect Dis* **16**: 352-354.

Kuzmin IV, Schwarz TM, Ilinykh PA, Jordan I, Ksiazek TG, Sachidanandam R, Basler CF and Bukreyev A (2017). Innate immune responses of bat and human cells to filoviruses: commonalities and distinctions. *J Virol* **91**: e02471-16.

Kwiecinski G and Griffiths T (1999). *Rousettus aegyptiacus*. *Mammalian species* **611**: 1-9.

Laing E, Yan L, Sterling S and Broder C (2016). A Luminex-based multiplex assay for the simultaneous detection of glycoprotein specific antibodies to ebolaviruses, marburgviruses, and henipaviruses. *Int J Infect Dis* **53**: 108-109.

Lee AK, Kulcsar KA, Elliott O, Khiabanian H, Nagle ER, Jones MEB, Amman BR, Sanchez-Lockhart M, Towner JS, Palacios G and Rabadan R (2015). De novo transcriptome reconstruction and annotation of the Egyptian rousette bat. *BMC Genomics* **16**:1033.

Leroy EM, Baize S, Volchkov VE, Fisher-Hoch SP, Georges-Courbot MC, Lansoud-Soukate J, Capron M, Debre P, McCormick JB and Georges AJ (2000). Human asymptomatic Ebola infection and strong inflammatory response. *Lancet* **355**: 2210-2215.

Leroy EM, Baize S, Debre P, Lansoud-Soukate J and Mavoungou E (2001). Early immune responses accompanying human asymptomatic Ebola infections. *Clin Exp Immunol* **124**: 453-460.

Leroy EM, Baize S, Mavoungou E and Apetrei C (2002). Sequence analysis of the GP, NP, VP40 and VP24 genes of Ebola virus isolated from deceased, surviving and asymptomatically infected individuals during the 1996 outbreak in Gabon: comparative studies and phylogenetic characterization. *J Gen Virol* **83**: 67-73.

Leroy EM, Kumulungui B, Pourrut X, Rouquet P, Hassanin A, Yaba P, Delicat A, Paweska JT, Gonzales JP and Swanepoel R (2005). Fruit bats as reservoirs of Ebola virus. *Nature* **438**: 575-576.

Lindblad-Toh K, Garber M, Zuk O, Lin MF, Parker BJ, Washietl S, Kheradpour P, Ernst J, Jordan G, Mauceli E, Ward LD, Lowe CB, Holloway AK, Clamp M, Gnerre S, Alföldi J, Beal K, Chang J, Clawson H, Cuff J, Di Palma F, Fitzgerald S, Flicek P, Guttman M, Hubisz MJ, Jaffe DB, Jungreis I, Kent WJ, Kostka D, Lara M, Martins AL, Massingham T, Moltke I, Raney BJ, Rasmussen MD, Robinson J, Stark A, Vilella AJ, Wen J, Xie X, Zody MC, Broad Institute Sequencing Platform and Whole Genome Assembly Team, Worley KC, Kovar CL, Muzny DM, Gibbs RA, Baylor College of Medicine Human Genome Sequencing Center Sequencing Team, Warren WC, Mardis ER, Weinstock GM, Wilson RK, Genome Institute at Washington University, Birney E, Margulies EH, Herrero J, Green ED, Haussler D, Siepel A, Goldman N, Pollard KS, Pedersen JS, Lander ES and Kellis M (2011). A high-resolution map of human evolutionary constraint using 29 mammals. *Nature* **478**: 476-482.

Lofts LL, Ibrahim MS, Negley DL, Hevey MC and Schmaljohn AL (2007). Genomic differences between guinea pig lethal and nonlethal Marburg virus variants. *J Infect Dis* **196**: S305-S312.

Lofts LL, Wells JB, Bavari S and Warfield KL (2011). Key genomic changes necessary for an *in vivo* lethal mouse marburgvirus variant selection process. *J Virol* **85**: 3905-3917.

Lopez L, Venteo A, Garcia M, Camuñas A, Ranz A, Garcia J, Sarraseca J, Anaya C and Rueda P (2009). Antigen-capture blocking enzyme-linked immunosorbent assay based on a baculovirus recombinant antigen to differentiate transmissable gastroenteritis virus from porcine respiratory coronavirus antibodies. *J Vet Diagn Invest* **21**: 598-608.

Lub MI, Sergeev AN, P'iankov OV, P'iankova OG, Petrishchenko VA and Kotliarov LA (1995). Certain pathogenetic characteristics of a disease in monkeys infected with the Marburg virus by an airborne route. *Vopr Virusol* **40**: 158-161.

Lucan RK, Bartonicka T, Benda P, Bilgin R, Jedlicka P, Nicolaou H, Reiter A, Shohdi WM, Salek M, Rerucha S, Uhrin M, Abi-Said M and Horacek I (2014). Reproductive seasonality of the Egyptian fruit bat (*Rousettus aegyptiacus*) at the northern limits of its distribution. *J Mammal* **95**: 1036-1042.

Luis AD, Hayman DT, O'Shea TJ, Cryan PM, Gilbert AT, Pulliam JR, Mills JN, Timonin ME, Willis CK, Cunningham AA, Fooks AR, Rupprecht CE, Wood JL and Webb CT (2013). A comparison of bats and rodents as reservoirs of zoonotic viruses: are bats special? *Proc Biol Sci* **280**: 20122753.

MacNeil A, Farnon EC, Wamala J, Okware S, Cannon DL, Reed ZD, Towner JS, Tappero JW, Lutwama J, Downing R, Nichol ST, Ksiazek TG and Rollin PE (2010). Proportion of deaths and clinical features in Bundibugyo Ebola virus infection, Uganda. *Emerg Infect Dis* **16**: 1969-1972.

MacNeil A, Farnon EC, Morgan OW, Gould P, Boehmer TK, Blaney DD, Wiersma P, Tappero JW, Nichol ST, Ksiazek TG and Rollin PE (2011a). Filovirus outbreak surveillance: lessons from Bundibugyo. *J Infect Dis* **204**: S761-S767.

MacNeil A, Reed ZD and Rollin PE (2011b). Serologic cross-reactivity of human IgM and IgG antibodies to five species of Ebola virus. *PLoS Negl Trop Dis* **5**: e1175.

Manicassamy B, Wang J, Rumschlag E, Tymen S, Volchkova V, Volchkov V and Rong L (2007). Characterization of Marburg virus glycoprotein in viral entry. *Virology* **358**: 79-88.

Marsh GA, de Jong C, Barr JA, Tachedjian M, Smith C, Middleton D, Yu M, Todd S, Foord AJ, Haring V, Payne J, Robinson R, Broz I, Crameri G, Field HE and Wang LF (2012). Cedar virus: a novel Henipavirus isolated from Australian bats. *PLoS Pathog* **8**: e1002836.

Martina JA, Daniotti JL and Maccioni HJF (1998). Influence of N-glycosylation and N-glycan trimming on the activity and intracellular traffic of GD3 synthase. *J Biol Chem* **273**: 3725-3731.

Martines RB, Ng DL, Greer PW, Rollin PE and Zaki SR (2015). Tissue and cellular tropism, pathology and pathogenesis of Ebola and Marburg viruses. *J Pathol* **235**: 153-174.

Martini GA (1969). Marburg agent disease: in man. *Trans R Soc Trop Med Hyg* **63**: 295-302.

Marzi A, Banadyga L, Haddock E, Thomas T, Shen K, Horne EJ, Scott DP, Feldmann H and Ebihara H (2016). A hamster model for Marburg virus infection accurately recapitulates Marburg hemorrhagic fever. *Sci Rep* **6**: 39214.

Maruyama T, Rodriguez LL, Jahrling PB, Sanchez A, Khan AS, Nichol ST, Peters CJ, Parren PW and Burton DR (1999). Ebola virus can be effectively neutralized by antibody produced in natural human infection. *J Virol* **73**: 6024-6030.

Mavrakis M, Kolesnikova L, Schoehn G, Becker S and Ruigrok RW (2002). Morphology of Marburg virus NP-RNA. *Virology* **296**: 300-307.

McBride GB (2005). A proposal for strength-of-agreement criteria for Lin's Concordance Correlation Coefficient. NIWA Client Report: HAM2005-062.

McElroy AK, Akondy RS, Davis CW, Ellebedy AH, Mehta AK, Kraft CS, Lyon GM, Ribner BS, Varkey J, Sidney J, Sette A, Campbell S, Stroher U, Damon I, Nichol ST, Spiropoulou CF, and Ahmed R (2015). Human Ebola virus infection results in substantial immune activation. *Proc Natl Acad Sci USA* **112**: 4719-4724.

Middleton DJ, Morrissy CJ, van der Heide BM, Russell GM, Braun MA, Westbury HA, Halpin K and Daniels PW (2007). Experimental Nipah virus infection in pteropid bats (*Pteropus poliocephalus*). *J Comp Pathol* **136**: 266-272.

Miller MR, McMinn RJ, Misra V, Schountz T, Muller MA, Kurth A and Munster VJ (2016). Broad and temperature independent replication potential of filoviruses on cells derived from old and new world bat species. *J Infect Dis* **214**: S297-S302.

Mire CE, Geisbert JB, Agans KN, Satterfield BA, Versteeg KM, Fritz EA, Feldmann H, Hensley LE and Geisbert TW (2014). Durability of a vesicular stomatitis virus-based Marburg virus vaccine in nonhuman primates. *PLoS ONE* **6**: e94355.

Misasi J, Chandran K, Yang JY, Considine B, Filone CM, Cote M, Sullivan N, Fabozzi G, Hensley L and Cunningham J (2012). Filoviruses require endosomal cysteine proteases for entry but exhibit distinct protease preferences. *J Virol* **86**: 3284-3292.

Mittler E, Kolesnikova L, Strecker T, Garten W and Becker S (2007). Role of the transmembrane domain of Marburg virus surface protein GP in assembly of the viral envelope. *J Virol* **81**: 3942-3948.

Mittler E, Kolesnikova L, Hartlieb B, Davey R and Becker S (2011). The cytoplasmic domain of Marburg virus GP modulates early steps of viral infection. *J Virol* **85**: 8188-8196.

Moe JB, Lambert RD and Lupton HW (1981). Plaque assay for Ebola virus. *J Clin Microbiol* **13**: 791-793.

Mühlberger E, Lötfering B, Klenk HD and Becker S (1998). Three of the four nucleocapsid proteins of Marburg virus, NP, VP35, and L, are sufficient to mediate replication and transcription of Marburg virus-specific monocistronic minigenomes. *J Virol* **72**: 8756-8764.

Mutere FA (1968). The breeding biology of the fruit bat *Rousettus aegyptiacus E. Geoffroy* living at 0 degrees 22'S. *Acta Trop* **25**: 97-108.

Nakayama E, Yokoyama A, Miyamoto H, Igarashi M, Kishida N, Matsuno K, Marzi A, Feldmann H, Ito K, Saijo M and Takada A (2010). Enzyme-linked immunosorbent assay for detection of filovirus species-specific antibodies. *Clin Vaccine Immunol* **17**: 1723-1728.

Nakayama E, Tomabechi D, Matsuno K, Kishida N, Yoshida R, Feldmann H and Takada A (2011). Antibody-dependent enhancement of Marburg virus infection. J Infect Dis 204: S978-S985.

Nakounne E, Selekon B and Morvan J (1990). Microbiological surveillance: viral hemorrhagic fever in Central African Republic: current serological data in man. *Bulletin de la Societe de pathologie exotique* **93**: 340-347.

Natesan M, Jensen SM, Keasey SL, Kamata T, Kuehne IE, Stonier SW, Lutwama JJ, Lobel L, Dye JM and Ulrich RG (2016). Human survivors of disease outbreaks caused by Ebola or Marburg virus exhibit cross-reactive and long-lived antibody responses. *Clin Vaccine Immunol* **23**: 717-724.

Nelson RJ, Demas GE, Klein SL and Kriegsfield LJ (2000). Seasonal patterns of stress, immune function and disease. Cambridge University Press: New York, pp 89-114.

Niikura M, Ikegami T, Saijo M, Kurata T, Kurane I and Morikawa S (2003). Analysis of linear B-cell epitopes of the nucleoprotein of Ebola virus that distinguish Ebola virus subtypes. *Clin Diagn Lab Immunol* **10**: 83-87.

Nikiforov VV, Turovskii II, Kalinin PP, Akinfeeva LA, Katkova LR, Barmin VS, Riabchikova EI, Popkova NI, Shestopalov AM, Nazarov VP, Vedishchev SV and Netesov SV (1994). A case of a laboratory infection with Marburg fever. *Zh Mikrobiol Epidemiol Immunobiol* **3**: 104-106.

Noda T, Sagara H, Suzuki E, Takada A, Kida H and Kawaoka Y (2002). Ebola virus VP40 drives the formation of virus-like filamentous particles along with GP. *J Virol* **76**: 4855-4865.

Noda T, Halfmann P, Sagara H and Kawaoka Y (2007). Regions in Ebola virus VP24 that are important for nucleocapsid formation. *J Infect Dis* **196**: S247-S250.

Ogawa H, Miyamoto H, Ebihara H, Ito K, Morikawa S, Feldmann H and Takada A (2011). Detection of all known filovirus species by reverse transcription-polymerase chain reaction using a primer set specific for the viral nucleoprotein gene. *J Virol Methods* **171**: 310-313.

Okware S, Katongole-Mbidde E, Downing R, Tappero JW, Zaki SR, Ksiazek TG, Nichol ST and Rollin PE (2009). Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathog* **5**: e1000536.

Omatsu T, Bak EJ, Ishii Y, Kyuwa S, Tohya Y, Akashi H and Yoshikawa Y (2008). Induction and sequencing of Rousette bat interferon alpha and beta genes. *Vet Immunol Immunopathol* **124**: 169-176.

O'Shea TJ, Cryan PM, Cunningham AA, Fooks AR, Hayman DTS, Luis AD, Peel AJ, Plowright RK and Woon JLN (2014). Bat flight and zoonotic viruses. *Emerg Infect Dis* **20**: 741-745.

Oswald WB, Geisbert TW, Davis KJ, Geisbert JB, Sullivan NJ, Jahrling PB, Parren PW an Burton DR (2007). Neutralising antibody fails to impact the course of Ebola virus infection in monkeys. *PLoS Pathog* **3**: e9.

Page A, Volchkova VA, Reid SP, Mateo M, Bagnaud-Baule A, Nemirov K, Shurtleff AC, Lawrence P, Reynard O, Ottmann M, Lotteau V, Biswal SS, Thimmulappa RK, Bavari S and Volchkov VE (2014). Marburg virus hijacks nrf2-dependent pathway by targeting nrf2-negative regulator Keap1. *Cell Rep* **6**: 1026-1036.

Panning M, Laue T, Olschlager S, Eickmann M, Becker S, Raith S, Georges Courbot M-C, Nilsson M, Gopal R, Lundkvist A, di Caro A, Brown D, Meyer H, Lloyd G, Kümmerer BM, Günther S and Drosten C (2007). Diagnostic reverse-transcription polymerase chain reaction kit for filoviruses based on the strain collections of all European biosafety level 4 laboratories. *J Infect Dis* **196**: S199-S204.

Papenfuss AT, Baker ML, Feng ZP, Tachedjian M, Crameri G, Cowled C, Ng J, Janardhana V, Field HE and Wang LF (2012). The immune gene repertoire of an important viral reservoir, the Australian black flying fox. *BMC Genomics* **13**: 261.

Parker J, Tsagkogeorga G, Cotton JA, Liu Y, Provero P, Stupka E and Rossiter SJ (2013). Genome-wide signatures of convergent evolution in echolocating mammals. *Nature* **502**: 228-231.

Parren PW, Geisbert TW, Maruyama T, Jahrling PB and Burton DR (2002). Pre- and postexposure prophylaxis of Ebola virus infection in an animal model by passive transfer of a neutralizing human antibody. *J Virol* **76**: 6408-6412.

Pavlovich SS, Lovett SP, Koroleva G, Guito JC, Arnold CE, Nagle ER, Kulcsar K, Lee A, Thibaud-Nissen F, Hume AJ, Mühlberger E, Uebelhoer LS, Towner JS, Rabadan R, Sanchez-Lockhart M, Kepler TB and Palacios G (2018). The Egyptian rousette genome reveals unexpected features of bat antiviral immunity. *Cell* **173**: 1-13.

Paweska JT, Burt FJ and Swanepoel R (2005). Validation of IgG-sandwich and IgM-capture ELISA for the detection of antibody to Rift Valley fever virus in humans. *J Virol Methods* **124**: 173-181.

Paweska JT, Jansen van Vuren P and Swanepoel R (2007). Validation of an indirect ELISA based on a recombinant nucleocapsid protein of Rift Valley fever virus for the detection of IgG antibody in humans. *J Virol Methods* **146**: 119-124.

Paweska JT, Jansen van Vuren P, Masumu J, Leman PA, Grobbelaar AA, Birkhead M, Clift C, Swanepoel R and Kemp A (2012). Virological and serological findings in *Rousettus aegyptiacus* experimentally inoculated with Vero cells-adapted Hogan strain of Marburg virus. *PLoS ONE* **7**: e45479.

Paweska JT, Jansen van Vuren P, Fenton KA, Graves K, Grobbelaar AA, Moolla N, Leman P, Weyer J, Storm N, McCulloch SD, Scott TP, Markotter W, Odendaal L, Clift SJ, Geisbert TW, Hale MJ and Kemp A (2015). Lack of Marburg virus transmission from experimentally infected to susceptible in-contact Egyptian fruit bats. *J Infect Dis* **212**: S109-S118.

Paweska JT, Storm N, Grobbelaar AA, Markotter W, Kemp A and Jansen van Vuren P (2016). Experimental inoculation of Egyptian fruit bats (*Rousettus aegyptiacus*) with Ebola virus. *Viruses* **8**: 29.

Paweska JT, Jansen van Vuren P, Kemp A, Storm N, Grobbelaar A, Wiley MR, Palacios G and Markotter W (2018). Marburg virus infection in Egyptian rousette bats, South Africa, 2013–2014. *Emerg Infect Dis* **24**: 6.

Peel AJ, Pulliam JR, Luis AD, Plowright RK, O'Shea TJ, Hayman DT, Wood JL, Webb CT and Restif O (2014). The effect of seasonal birth pulses on pathogen persistence in wild mammal populations. *Proc Biol Sci* **281**: 20132962.

Penzhorn BL and Rautenbach IL (1988). Reproduction of the Egyptian fruit bat *Rousettus aegyptiacus* in the southern tropics. *South African Journal of Nature* **18**: 88-92.

Peters C, Isaza R, Heard DJ, Davis RD, Moore SM and Briggs DJ (2004). Vaccination of Egyptian fruit bats (*Rousettus aegyptiacus*) with monovalent inactivated rabies vaccine. *J Zoo Wildl Med* **35**: 55-59.

Peters CJ (2005). Marburg and Ebola virus hemorrhagic fevers. In: Mandell GL, Bennett JE and Dolin R, editors. Mandell, Douglas and Bennett's principles and practice of infectious diseases. Elsevier Inc.: Philadelphia, pp 2057-2059.

Pigott DM, Golding N, Mylne A, Huang Zhi, Weiss DJ, Brady OJ, Kraemer MUG and Hay SI (2015). Mapping the zoonotic niche of Marburg virus disease in Africa. *Trans R Soc Trop Med Hyg* **109**: 366-378.

Plowright RK, Field HE, Smith C, Divljan A, Palmer C, Tabor G, Daszak P and Foley JE (2008). Reproduction and nutritional stress are risk factors for Hendra virus infection in little red flying foxes (*Pteropus scapulatus*). *Proc Biol Sci* **275**: 861-869.

Plowright RK, Peel AJ, Streicker DG, Gilbert AT, McCallum H, Wood J, Baker ML and Restif O (2016). Transmission or within-host dynamics driving pulses of zoonotic viruses in reservoir-host populations. *PLoS Negl Trop Dis* **10**: e0004796.

Pourrut X, Delicat A, Rollin PE, Ksiazek TG, Gonzalez JP and Leroy E (2007). Spatial and temporal patterns of Zaire ebolavirus antibody prevalence in the possible reservoir bat species. *J Infect Dis* **196**: S176-S183.

Pourrut X, Souris M, Towner JS, Rollin PE, Nichol ST, Gonzalez JP and Leroy E (2009). Large serological survey showing co-circulation of Ebola and Marburg viruses in Gabonese bat populations, and a high seroprevalence of both viruses in *Rousettus aegyptiacus. BMC Infect Dis* **9**: 159.

Prehaud C, Hellebrand E, Coudrier D, Volchkov VE, Volchkova VA, Feldmann H, Le Guenno B and Bouloy M (1998). Recombinant Ebola virus nucleoprotein and glycoprotein (Gabon 94 strain) provide new tools for the detection of human infections. *J Gen Virol* **79**: 2565-2572.

Randall RE and Goodbourn S (2008). Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol* **89**: 1-47.

Rao MKK, Kapila K and Gupta RM (1997). Enzyme linked immunosorbent assays revisited. *Med J Armed Forces India* **53**: 45-51.

Reid SP, Leung LW, Hartman AL, Martinez O, Shaw ML, Carbonelle C, Volchkov VE, Nichol ST and Basler CF (2006). Ebola virus VP24 binds karyopherin a1 and blocks STAT1 nuclear accumulation. *J Virol* **80**: 5156-5167.

Reynes JM, Molia S, Audry L, Hout S, Ngin S, Walston J and Bourhy H (2004). Serologic evidence of lyssavirus infection in bats, Cambodia. *Emerg Infect Dis* **10**: 2231-2234.

Rieger T, Kerber R, El Halas H, Pallasch E, Duraffour S, Günther S and Ölschläger S (2016). Evaluation of RealStar reverse transcription polymerase chain reaction kits for filovirus detection in the laboratory and field. *J Infect Dis* **214**: S243-S249.

Rimoin AW, Lu K, Bramble MS, Steffen I, Doshi RH, Hoff NA, Mukadi P, Nicholson BP, Alfonso VH, Olinger G, Sinai C, Yamamoto LK, Ramirez CM, Okitolonda Wemakoy E, Kebela Illunga B, Pettitt J, Logue J, Bennett RS, Jahrling P, Heymann DL, Piot P, Muyembe-Tamfum JJ, Hensley LE and Simmons G (2018). Ebola virus neutralizing antibodies detectable in survivors of Yambuku, Zaire outbreak 40 years after infection. *J Infect Dis* **217**: 223-231.

Roddy P, Thomas SL, Jeffs B, Nascimento Folo P, Pablo Palma P, Moco Henrique B, Villa L, Paixao Damiao Machado F, Bernal O, Jones SM, Strong JE, Feldmann H and Borchert M (2010). Factors associated with Marburg hemorrhagic fever: analysis of patient data form Uige, Angola. *J Infect Dis* **201**: 1909-1918.

Rosano GL and Ceccarelli EA (2014). Recombinant protein expression in *Escherichia coli*: advances and challenges. *Front Microbiol* **5**: 172.

Rougeron V, Feldmann H, Grard G, Becker S and Leroy EM (2015). Ebola and Marburg haemorrhagic fever. *J Clin Virol* **64**: 111-119.

Rouquet P, Froment JM, Bermejo M, Kilbourn A, Karesh W, Reed P, Kumulungui B, Yaba P, Delicat A, Rollin PE and Leroy EM (2005). Wild animal mortality monitoring and human Ebola outbreaks, Gabon and Republic of Congo, 2001–2003. *Emerg Infect Dis* **11**: 283-290.

Ruibal PL, Oestereich A, Ludtke B, Becker-Ziaja DM, Wozniak R, Kerber M, Korva M, Cabeza-Cabrerizo JA, Bore FR, Koundouno FR, Duraffour S, Weller R, Thorenz A, Cimini E, Viola D, Agrati C, Repits J, Afrough B, Cowley LA, Ngabo D, Hinzmann J, Mertens M, Vitoriano I, Logue CH, Boettcher JP, Pallasch E, Sachse A, Bah A, Nitzsche K, Kuisma E, Michel J, Holm T, Zekeng EG, Garcia-Dorival I, Wolfel R, Stoecker K, Fleischmann E, Strecker T, Di Caro A, Avsic-Zupanc T, Kurth A, Meschi S, Mely S, Newman E, Bocquin A, Kis Z, Kelterbaum A, Molkenthin P, Carletti F, Portmann J, Wolff S, Castilletti C, Schudt G, Fizet A, Ottowell LJ, Herker E, Jacobs T, Kretschmer B, Severi E, Ouedraogo N, Lago M, Negredo A, Franco L, Anda P, Schmiedel S, Kreuels B, Wichmann D, Addo MM, Lohse AW, De Clerck H, Nanclares C, Jonckheere S, Van Herp M, Sprecher A, Xiaojiang G, Carrington M, Miranda O, Castro CM, Gabriel M, Drury P, Formenty P, Diallo B, Koivogui L, Magassouba N, Carroll MW, Gunther S and Munoz-Fontela C (2016). Unique human immune signature of Ebola virus disease in Guinea. *Nature* **533**: 100-104.

Ryabchikova EI, Kolesnikova LV and Netesov SV (1999). Animal pathology of filoviral infections. *Curr Top Microbiol Immunol* **235**: 145-173.

Ryan EL, Hollingworth R and Grand RJ (2016). Activation of the DNA damage response by RNA viruses. *Biomolecules* **6**: 2.

Sadler AJ and Williams BRG (2008). Interferon-inducible antiviral effectors. *Nat Rev Immunol* **8**: 559-568.

Saijo M, Niikura M, Morikawa S, Ksiazek TG, Meyer RF, Peters CJ and Kurane I (2001a). Enzyme-linked immunosorbent assays for detection of antibodies to Ebola and Marburg viruses using recombinant nucleoproteins. *J Clin Microbiol* **39**: 1-7.

Saijo M, Niikura M, Morikawa S and Kurane I (2001b). Immunofluorescence method for detection of Ebola virus immunoglobulin G, using HeLa cells which express recombinant nucleoprotein. *J Clin Microbiol* **39**: 776-778.

Saijo M, Niikura M, Maeda A, Sata T, Kurata T, Kurane I and Morikawa S (2005). Characterization of monoclonal antibodies to Marburg virus nucleoprotein (NP) that can be used for NP-capture enzyme-linked immunosorbent assay. *J Med Virol* **76**: 111-118.

Saijo M, Niikura M, Ikegami T, Kurane I, Kurata T and Morikawa S (2006a). Laboratory diagnostic systems for Ebola and Marburg hemorrhagic fevers developed with recombinant proteins. *Clin Vaccine Immunol* **13**: 444-451.

Saijo M, Georges-Courbot MC, Fukushi S, Mizutani T, Philippe M, Georges AJ, Kurane I and Morikawa S (2006b). Marburgvirus nucleoprotein-capture enzyme-linked immunosorbent assay using monoclonal antibodies to recombinant nucleoprotein: detection of authentic Marburg virus. *Jpn J Infect Dis* **59**: 323-325.

Sambrook J and Russel DW (2001). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press: New York, pp 82-85.

Sanchez A, Kiley MP, Klenk HD and Feldmann H (1992). Sequence analysis of the Marburg virus nucleoprotein gene: comparison to Ebola virus and other non-segmented negative-strand RNA viruses. *J Gen Virol* **73**: 347-357.

Sanchez A, Kiley MP, Holloway BP and Auperin DD (1993). Sequence analysis of the Ebola virus genome: organization, genetic elements, and comparison with the genome of Marburg virus. *Virus Res* **29**: 215-240.

Sanchez A, Trappier SG, Ströher U, Nichol S, Bowen MD and Feldmann H (1998). Variation in the glycoprotein and VP35 genes of Marburg virus strains. *Virology* **240**: 138-146.

Sanchez A, Ksiazek TG, Rollin PE, Miranda ME, Trappier SG, Khan AS, Peters CJ and Nichol ST (1999). Detection and molecular characterization of Ebola viruses causing disease in human and nonhuman primates. *J Infect Dis* **179**: S164-S169.

Sanchez A, Kukwiya M, Bausch D, Mahanty S, Sanchez AJ, Wagoner KD and Rollin PE (2004). Analysis of human peripheral blood samples from fatal and nonfatal cases of Ebola (Sudan) hemorrhagic fever: cellular responses, viral load and nitric oxide levels. *J Virol* **78**: 10370-10377.

Schlee M and Hartmann G (2016). Discriminating self from non-self in nucleic acid sensing. *Nat Rev Immunol* **16**: 566-580.

Schountz T (2014). Immunology of bats and their viruses: challenges and opportunities. *Viruses* **6**: 4880-4901.

Schountz T, Baker ML, Butler J and Munster V (2017). Immunological control of viral infections in bats and the emergence of viruses highly pathogenic to humans. *Front Immunol* **8**: 1098.

Schuh AJ, Amman BR, Jones MEB, Sealy TK, Uebelhoer LS, Spengler JR, Martin BE, Coleman-McCray JD, Nichol ST and Towner JS (2017a). Modelling filovirus maintenance in nature by experimental transmission of Marburg virus between Egyptian rousette bats. *Nat Commun* **8**: 14446.

Schuh AJ, Amman BR, Sealy TK, Spengler JR, Nichol ST and Towner JS (2017b). Egyptian rousette bats maintain long-term protective immunity against Marburg virus infection despite diminished antibody levels. *Sci Rep* **7**: 8763.

Sedgwick P (2012). Pearson's correlation coefficient. *BMJ* **345**: e4483.

Seim I, Fang X, Xiong Z, Lobanov AV, Huang Z, Ma S, Feng Y, Turanov AA, Zhu Y, Lenz TL, Gerashchenko MV, Fan D, Hee Yim S, Yao X, Jordan D, Xiong Y, Ma Y, Lyapunov AN,

Chen G, Kulakova OI, Sun Y, Lee SG, Bronson RT, Moskalev AA, Sunyaev SR, Zhang G, Krogh A, Wang J and Gladyshev VN (2013). Genome analysis reveals insights into physiology and longevity of the Brandt's bat *Myotis brandtii*. *Nat Commun* **4**: 2212.

Shaw TI, Srivastava A, Chou W, Liu L, Hawkinson A, Glenn TC, Adams R and Schountz T (2012). Transcriptome sequencing and annotation for the Jamaican fruit bat (*Artibeus jamaicensis*). *PLoS ONE* **7**: 48472.

Shedlock DJ, Bailey MA, Popernack PM, Cunningham JM, Burton DR and Sullivan NJ (2010). Antibody-mediated neutralization of Ebola virus can occur by two distinct mechanisms. *Virology* **401**: 228-235.

Shen YY, Liang L, Zhu ZH, Zhou WP, Irwin DM and Zhang YP (2010). Adaptive evolution of energy metabolism genes and the origin of flight in bats. *Proc Natl Acad Sci USA* **107**: 8666-8671.

Sherwood LJ, Osborn LE, Carrion R Jr, Patterson JL and Hayhurst A (2007). Rapid assembly of sensitive antigen-capture assays for Marburg virus, using in vitro selection of llama single-domain antibodies, at biosafety level 4. *J Infect Dis* **196**: S213-S219.

Shimojima M, Takada A, Ebihara H, Neumann G, Fujioka K, Irimura T, Jones S, Feldmann H and Kawaoka Y (2006). Tyro3 family-mediated cell entry of Ebola and Marburg viruses. *J Virol* **80**: 10109-10116.

Shurtleff AC, Biggins JE, Keeney AE, Zumbrun EE, Bloomfield HA, Kuehne A, Audet JL, Alfson KJ, Griffiths A, Olinger GG and Bavari S (2012). Standardization of the filovirus plaque assay for use in preclinical studies. *Viruses* **4**: 3511-3530.

Siegenthaler W (2007). Differential diagnosis in internal medicine: from symptom to diagnosis. Thieme: Stuttgart, Germany, p 171.

Siegert R, Shu HL, Slenczka W, Peters D and Müller G (1968a). The aetiology of an unknown human infection transmitted by monkeys [preliminary communication]. *Ger Med Mon* **13**: 1-2.

Siegert R, Shu HL and Slenczka W (1968b). Isolation and identification of the Marburg virus. *Dtsch Med Wochenschr* **93**: 604-612.

Siegert R (1972). Marburg virus. In: Virology Monograph. Springer-Verlag: New York, pp 98-153.

Silver S (2015). Laboratory-acquired lethal infections by potential bioweapons pathogens including Ebola in 2014. *FEMS Microbiol Lett* **362**: 1-6.

Simmons G, Wool-Lewis RJ, Baribaud F, Netter RC and Bates P (2002). Ebola virus glycoproteins induce global surface protein down-modulation and loss of cell adherence. *J Virol* **76**: 2518-2528.

Skripchenko AA, Riabchikova EI, Vorontsova LA, Shestopalov AM and Viazunov SA (1994). Marburg virus and mononuclear phagocytes: study of interactions. *Vopr Virusol* **39**: 214-218.

Smith DH, Johnson BK, Isaacson M, Swanepoel R, Johnson KM, Killey M, Bagshawe A, Siongok T and Keruga WK (1982). Marburg virus disease in Kenya. *Lancet* **1**: 816-820.

So HS, Yoon HS, Choi DY, Kwon YS, Sung JH, Lee TG, Park ES, Cho HS, Lee BM, Cho JM and Ryu WS (1997). Effect of a novel saponin adjuvant derived from *Quillaja saponaria* on the immune response to recombinant hepatitis B surface antigen. *Mol Cells* **7**: 178-186.

Sobarzo A, Perelman E, Groseth A, Dolnik O, Becker S, Lutwama JJ, Dye JM, Yavelsky V, Lobel L and Marks RS (2012). Profiling the native specific human humoral immune response to Sudan ebolavirus strain Gulu by chemiluminescence enzyme-linked immunosorbent assay. *Clin Vaccine Immunol* **19**: 1844-1852.

Sobarzo A, Groseth A, Dolnik O, Becker S, Lutwama JJ, Perelman E, Yavelsky V, Muhammad M, Kuehne AI, Marks RS, Dye JM and Lobel L (2013). Profile and persistence of the virus-specific neutralizing humoral immune response in human survivors of Sudan ebolavirus (Gulu). *J Infect Dis* **208**: 299-309.

Sobarzo A, Stonier SW, Herbert AS, Ochayon DE, Kuehne AI, Eskira Y, Fedida-Metula S, Tali N, Lewis EC, Egesa M, Cose S, Lutwama JJ, Yavelsky V, Dye JM and Lobel L (2016). Correspondence of neutralizing humoral immunity and CD4 T cell responses in long recovered Sudan virus survivors. *Viruses* **8**: 133.

Spearman C (1908). The method of right and wrong cases (constant stimuli) without Gauss's formulae. *Br J Psychol* **2**: 227-242.

Spickler AR (2010). Emerging and exotic diseases of animals. Iowa State University: Iowa, USA, p 310.

Stonier SW, Herbert AS, Kuehne AI, Sobarzo A, Habibulin P, Abramovitch Dahan CV, James RM, Egesa M, Cose S, Lutwama JJ, Lobel L and Dye JM (2017). Marburg virus survivor immune responses are Th1 skewed with limited neutralizing antibody responses. *J Exp Med* **214**: 2563.

Sulkin SE, Allen R, Sims R and Singh KV (1966). Studies of arthropod-borne virus infections in Chiroptera. IV. The immune response of the big brown bat (*Eptesicus f. fuscus*) maintained at various environmental temperatures to experimental Japanese B encephalitis virus infections. *Am J Trop Med Hyg* **15**: 418–427.

Sullivan NJ, Peterson M, Yang Z, Kong W, Duckers H, Nabel E and Nabel GJ (2005). Ebola virus glycoprotein toxicity is mediated by a dynamin-dependent protein-trafficking pathway. *J Virol* **79**: 547-553.

Swanepoel R, Leman PA, Burt FJ, Zachariades NA, Braack LE, Ksiazek TG, Rollin PE, Zaki SR and Peters CJ (1996). Experimental inoculation of plants and animals with Ebola virus. *Emerg Infect Dis* **2**: 321-325.

Swanepoel R, Smit SB, Rollin PE, Formenty P, Leman PA, Kemp A, Burt FJ, Grobbelaar AA, Croft J, Bausch DG, Zeller H, Leirs H, Braack LEO, Libande ML, Zaki S, Nichol ST, Ksiazek TG and Paweska JT (2007). Studies of reservoir hosts for Marburg virus. *Emerg Infect Dis* **13**: 1847-1851.

Swenson DL, Wang D, Luo M, Warfield KL, Woraratanadharm J, Holman DH, Dong JY and Pratt WD (2008). Vaccine to confer to nonhuman primates complete protection against multistrain Ebola and Marburg virus infections. *Clin Vaccine Immunol* **15**: 460-467.

Takada A, Watanabe S, Ito H, Okazaki K, Kida H and Kawaoka Y (2000). Downregulation of beta1 integrins by Ebola virus glycoprotein: implication for virus entry. *Virology* **278**: 20-26.

Takada A, Ebihara H, Jones S, Feldmann H and Kawaoka Y (2007a). Protective efficacy of neutralizing antibodies against Ebola virus infection. *Vaccine* **25**: 993-999.

Takada A, Ebihara H, Feldmann H, Geisbert TW and Kawaoka Y (2007b). Epitopes required for antibody-dependent enhancement of Ebola virus infection. *J Infect Dis* **196**: S347-S356.

Taniguchi S, Sayama Y, Nagata N, Ikegami T, Miranda ME, Watanabe S, Iizuka I, Fukushi S, Mizutani T, Ishii Y, Saijo M, Akashi H, Yoshikawa Y, Kyuwa S and Morikawa S (2012). Analysis of the humoral immune responses among cynomolgus macaques naturally infected with Reston virus during the 1996 outbreak in the Philippines. *BMC Vet Res* **8**: 189.

Timen A, Koopmans MP, Vossen AC, van Doornum GJ, Gunther, S, van den Berkmortel F, Verduin KM, Dittrich S, Emmerich P, Osterhaus AD, van Dissel JT and Coutinho RA (2009). Response to imported case of Marburg hemorrhagic fever, the Netherlands. *Emerg Infect Dis* **15**: 1171-1175.

Towner JS, Khristova ML, Sealy TK, Vincent MJ, Erickson BR, Bawiec DA, Hartman AL, Comer JA, Zaki SR, Stroher U, Gomes da Silva F, del Castillo F, Rollin PE, Ksiazek TG and Nichol ST (2006). Marburgvirus genomics and association with a large hemorrhagic fever outbreak in Angola. *J Virol* **80**: 6497-6516.

Towner JS, Pourrut X, Albariño CG, Nkogue CN, Bird BH, Grard G, Ksiazek TG, Gonzalez JP, Nichol ST and Leroy EM (2007). Marburg virus infection detected in a common African bat. *PLoS ONE* **2**: e764.

Towner JS, Amman BR, Sealy TK, Carrol SA, Comer JA, Kemp A, Swanepoel R, Paddock CD, Balinandi S, Khristova ML, Formenty PB, Albariño CG, Miller DM, Reed ZD, Kayiwa JT, Mills JN, Cannon DL, Greer PW, Byaruhanga E, Farnon EC, Atimnedi P, Okware S, Katongole-Mbidde E, Downing R, Tappero JW, Zaki SR, Ksiazek TG, Nichol ST and Rollin PE (2009). Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathog* **5**: e1000536.

Truant AL, Regnery RL and Kiley MP (1983). Development of an immunofluorescence focus assay for Ebola virus. *J Clin Microbiol* **18**: 416-419.

Turmelle A, Ellison J, Mendonca M and McCracken F (2010). Histological assessment of cellular immune response to the phytohemagglutinin skin test in Brazilian free-tailed bats (*Tadarida brasiliensis*). *J Comp Physiol B* **180**: 1155-1164.

Valmas C, Grosch MN, Schumann M, Olejnik J, Martinez O, Best SM, Krahling V, Basler CF and Mühlberger E (2010). Marburg virus evades interferon responses by a mechanism distinct from Ebola virus. *PLoS Pathog* **6**: e1000721.

Valmas C and Basler CF (2011). Marburg virus VP40 antagonizes interferon signalling in a species-specific manner. *J Virol* **85**: 4309-4317.

Vanessa NR and Mattias B (2012). Infection control during filoviral hemorrhagic fever outbreaks. *J Glob Infect Dis* **4**: 69-74.

Volchkov VE, Feldmann H, Volchkova VA and Klenk HD (1998). Processing of the Ebola virus glycoprotein by the proprotein convertase furin. *Proc Natl Acad Sci USA* **95**: 5762-5767.

Volchkov VE, Chepurnov AA, Volchkova VA, Ternovoj VA and Klenk HD (2000). Molecular characterization of guinea pig–adapted variants of Ebola virus. *Virology* **277**: 147-155.

Warfield KL, Bradfute SB, Wells J, Lofts L, Cooper MT, Alves DA, Reed DK, Van Tongeren SA, Mech CA and Bavari S (2009). Development and characterization of a mouse model for Marburg hemorrhagic fever. *J Virol* **83**: 6404-6415.

Watt A, Moukambi F, Banadyga L, Groseth A, Callison J, Herwig A, Ebihara H, Feldmann H and Hoenen T (2014). A novel lifecycle modelling system for Ebola virus shows a genome length-dependent role of VP24 on virus infectivity. *J Virol* **88**: 10511-10524.

Weik M, Modrof J, Klenk HD, Becker S and Mühlberger E (2002). Ebola virus VP30-mediated transcription is regulated by RNA secondary structure formation. *J Virol* **76**: 8532-8539.

Weingartl HM, Nfon C and Kobinger G (2013). Review of Ebola virus infections in domectic animals. *Dev Biol (Basel)* **135**: 211-218.

Weissenhorn W, Carfi A, Lee KH, Skehel JJ and Wiley DC (1998). Crystal structure of the Ebola virus membrane fusion subunit, GP_2, from the envelope glycoprotein ectodomain. *Mol Cell* **2**: 605-616.

Wellehan JFX Jr, Green LG, Duke DG, Bootorabi S, Heard DJ, Klein PA and Jacobson ER (2009). Detection of specific antibody responses to vaccination in variable flying foxes (*Pteropus hypomelanus*). *Comp Immunol Microbiol* **32**: 379–394.

Wendelboe AM, Van Rie A, Salmaso S and Englund JA (2005). Duration of immunity against pertussis after natural infection or vaccination. *Pediatr Infect Dis J* **25**: S58-S61.

Wenigenrath J, Kolesnikova L, Hoenen T, Mittler E and Becker S (2010). Establishment and application of an infectious virus-like particle system for Marburg virus. *J Gen Virol* **91**: 1325-1334.

Wilson JA, Hevey M, Bakken R, Guest S, Bray M, Schmaljohn AL and Hart MK (2000). Epitopes involved in antibody-mediated protection from Ebola virus. *Science* **287**: 1664-1666.

Wong G, Kobinger GP and Qiu X (2014). Characterization of immune responses in Ebola virus infections. *Expert Rev Clin Immunol* **10**:781-790.

World Health Organisation (1978a). Ebola haemorrhagic fever in Sudan, 1976. *Bull World Health Organ* **56**: 247-270.

World Health Organisation (1978b). Ebola haemorrhagic fever in Zaire, 1976. *Bull World Health Organ* **56**: 271-293.

World Health Organisation (2007). Marburg haemorrhagic fever, Uganda. *Wkly Epidemiol Rec* **82**: 297-298.

World Health Organisation (2012). Marburg haemorrhagic fever, Uganda – update. *Wkly Epidemiol Rec* **87**: 437-448.

World Health Organisation (2014). Marburg virus disease – Uganda. [Online]. Available: <http://www.who.int/csr/don/13-november-2014-marburg/en/> [Accessed 26 February 2015].

World Health Organisation (2016). Ebola virus disease situation report – 10 June 2016. [Online]. Available: <http://apps.who.int/iris/bitstream/handle/10665/208883/ebolasitrep_10Jun2016_eng.pdf;jsessionid=4CE0486557523CF55FF6BC1803A91BEE?sequence=1> [Accessed 5 November 2018].

World Health Organisation (2017). Marburg virus Disease - Uganda and Kenya. [Online]. Available: <http://www.who.int/csr/don/7-november-2017-marburg/en/> [Accessed 27 November 2017].

Wright PF, Nilsson E, van Rooij EMA, Lelenta M and Jeggo MH (1993). Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. *Rev Sci Tech Off Int Epiz* **12**: 435-450.

Xie J, Li Y, Shen X, Goh G, Zhu Y, Cui J, Wang L-F, Shi Z-L and Zhou P (2018). Dampened STING-dependent interferon activation in bats. *Cell Host & Microbe* **23**: 297-301.

Yaddanapudi K, Palacios G, Towner JS, Chen I, Sariol CA, Nichol ST and Lipkin WI (2006). Implication of a retrovirus-like glycoprotein peptide in the immunopathogenesis of Ebola and Marburg viruses. *FASEB J* **20**: 2519-2530.

Yang Z, Duckers HJ, Sullivan NJ, Sanchez A, Nabel EG and Nabel GJ (2000). Identification of the Ebola virus glycoprotein as the main viral determinant of vascular cell cytotoxicity and injury. *Nat Med* **6**: 886.

Yang X-L, Zhang Y-Z, Jiang R-D, Guo H, Zhang W, Li B, Wang N, Wang L, Waruhiu C, Zhou J-H, Li S-Y, Daszak P, Wang L-F and Shi Z-L (2017). Genetically diverse filoviruses in *Rousettus* and *Eonycteris* spp. bats, China, 2009 and 2015. *Emerg Infect Dis* **23**: 482-486.

Yen B, Mulder LCF, Martinez O and Basler CF (2014). Molecular basis for ebolavirus VP35 suppression of human dendritic cell maturation. *J Virol* **88**: 12500-12510.

Yuan J, Zhang Y, Li J, Zhang Y, Wang L-F and Shi Z (2012). Serological evidence of ebolavirus infection in bats, China. *Virology J* **9**: 236.

Zhai J, Palacios G, Towner JS, Jabado O, Kapoor V, Venter M, Grolla A, Briese T, Paweska J, Swanepoel R, Feldmann H, Nichol ST and Lipkin WI (2007). Rapid molecular strategy for filovirus detection and characterization. *J Clin Microbiol* **45**: 224-226.

Zhang G, Cowled C, Shi Z, Huang Z, Bishop-Lilly KA, Fang X, Wynne JW, Xiong Z, Baker ML, Zhao W, Tachedjian M, Zhu Y, Zhou P, Jiang X, Ng J, Yang L, Wu L, Xiao J, Feng Y, Chen Y, Sun X, Zhang Y, Marsh GA, Crameri G, Broder CC, Frey KG, Wang L-F and Wang J (2013). Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. *Science* **339**: 456-460.

Zhang L, Li Q, Liu Q, Huang W, Nie J and Wang Y (2017). A bioluminescent imaging mouse model for Marburg virus based on a pseudovirus system. *Hum Vaccin Immunother* **13**: 1811-1817.

Zhou P, Cowled C, Marsh GA, Shi Z, Wang L-F and Baker ML (2011a). Type III IFN receptor expression and functional characterisation in the Pteropid bat, *Pteropus alecto*. *PLoS ONE* **6**: e25385.

Zhou P, Cowled C, Todd S, Crameri G, Virtue ER, Marsh GA, Klein R, Shi Z, Wang L-F and Baker LM (2011b). Type III IFNs in Pteropid bats: differential expression patterns provide evidence for distinct roles in antiviral immunity. *J Immunol* **186**: 3138-3147.

Zhou P, Tachedjian M, Wynne JW, Boyd V, Cui J, Smith I, Cowled C, Ng JH, Mok L, Michalski WP, Mendenhall IH, Tachedjian G, Wang LF and Baker ML (2016). Contraction of the type I IFN locus and unusual constitutive expression of IFN-alpha in bats. *Proc Natl Acad Sci USA* **113**: 2696-2701.

COMMUNICATIONS RELATED TO THIS THESIS

Storm N, Jansen van Vuren P, Markotter W and Paweska JT (2018). Antibody responses to Marburg virus in Egyptian rousette bats and their role in protection against infection. *Viruses* **10**: 73.

Paweska JT, Jansen van Vuren P, Fenton KA, Graves K, Grobbelaar AA, Moolla N, Leman P, Weyer J, Storm N, McCulloch SD, Scott TP, Markotter W, Odendaal L, Clift SJ, Geisbert TW, Hale MJ and Kemp A (2015). Lack of Marburg virus transmission from experimentally infected to susceptible in-contact Egyptian fruit bats. *J Infect Dis* **212**: S109-S118.

Scientific meetings and forums

Oral presentation: Storm N, Jansen van Vuren P, Markotter W and Paweska JT. The role of passive and acquired humoral immunity in the maintenance of Marburg virus in *Rousettus aegyptiacus* bat colonies. 9th International Filovirus Symposium, Marburg, Germany, September 13-16, 2017.

Oral presentation: Storm N, Jansen van Vuren P, McCulloch SD, Markotter W and Paweska JT. Serological responses of *Rousettus aegyptiacus* to Marburg virus. National Institute for Communicable Diseases Research Forum, Sandringham, South Africa, February 22, 2017.

Oral presentation: Storm N, Jansen van Vuren P, Markotter W and Paweska JT. Humoral immune responses of *Rousettus aegyptiacus* to Marburg virus. The 4th International One Health Congress & 6th Biennial Congress of the International Association for Ecology and Health, Melbourne, Australia, December 3-7, 2016.

Poster presentation: Storm N, Jansen van Vuren P, Markotter W and Paweska JT. Humoral immune responses of *Rousettus aegyptiacus* to the glycoprotein, nucleoprotein and structural proteins of Marburg virus. International Bat Research Conference, Durban, KwaZulu Natal, South Africa, July 31 - August 5, 2016.

APPENDIX A - ETHICS APPROVAL CERTIFICATES AND PERMITS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 2

PROJECT TITLE	**Disease ecology of pathogens in African bat species: Development and validation of serological assays for the diagnosis, surveillance and monitoring of immune responses of filovirus infection in humans and animals**
PROJECT NUMBER	**EC056-14 (Amendment 2)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Ms. N Storm**

STUDENT NUMBER (where applicable)	**U_2818 9869**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES/SAMPLES	***Rousettus aegyptiacus***
NUMBER OF ANIMALS	**670 sera from previously approved applications**
Approval period to use animals for research/testing purposes	**June 2018 – June 2019**
SUPERVISOR	**Prof. W Markotter**

KINDLY NOTE:

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

APPROVED	Date 16 July 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

Extension No. 2

PROJECT TITLE	**Re-infection of previously exposed *Rousettus aegyptiacus* bats with Marburg virus**
PROJECT NUMBER	**H018-16**
RESEARCHER/PRINCIPAL INVESTIGATOR	**N Storm**

STUDENT NUMBER (where applicable)	**U-281 898 69**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

Please provide a copy from DAFF that the facility comply to a BSL4 facility

ANIMAL SPECIES	**Fruit bats**
NUMBER OF SAMPLES	**32 approved; 17 used**
Approval period to use animals for research/testing purposes	**June 2018 – June 2019**
SUPERVISOR	**Prof. W Markotter**

KINDLY NOTE:

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

APPROVED	Date 13 June 2018
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Private Bag X138, Pretoria, 0001
Delpen Building, c/o Annie Botha & Union Street, Riviera, 0084

From: Directorate Animal Health
Tel: 012 319 7532
Fax: 012 319 7470
E-mail: HerryG@daff.gov.za
Enquiries: Mr. Herry Gololo
Our Ref: 12/11/1/1/13
Your Ref No : Appendix 2

Prof Janusz Paweska and Dr Wanda Markotter
NICD and University of Pretoria

Dear Prof Paweska and Dr. Markotter

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 16 November 2012, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;

2. Colonies to be established at the CEZD, NICD;

3. Animal welfare to be in compliance with NSPCA requirements

Title of research/study: Establishing an experimental *Rousettus aegyptiacus* and *Epomorphorus* spp colony

Researcher (s): Prof Paweska and Dr Wanda Markotter

Institution: NICD

Your Ref./ Project Number: Appendix 2

Our ref Number: 12/11/1/1/13

Kind regards

Dr W. F. UNGERER

DIRECTOR: ANIMAL HEALTH (Acting)

Date: 14/12/2012.

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Private Bag X138, Pretoria, 0001
Delpen Building, c/o Annie Botha & Union Street, Riviera, 0084

From: Directorate Animal Health
Tel: 012 319 7532
Fax: 012 319 7470
E-mail: HerryG@daff.gov.za
Enquiries: Mr. Herry Gololo
Our Ref: 12/11/1/1/13
Your Ref No : Appendix 6

Prof Janusz Paweska and Dr Wanda Markotter
NICD and University of Pretoria

Dear Prof Paweska and Dr. Markotter

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 16 November 2012, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;

2. The entire research study must be performed at the CEZD, NICD and no samples may leave the facility.

Title of research/study: Development of new diagnostic assays for filoviruses
Researcher (s): Prof Paweska and Dr Wanda Markotter
Institution: NICD

Your Ref./ Project Number: Appendix 6

Our ref Number: 12/11/1/1/13

Kind regards

DIRECTOR: ANIMAL HEALTH

Date: 2013 -02- 04

agriculture,
forestry & fisheries

Department:
Agriculture, Forestry and Fisheries
REPUBLIC OF SOUTH AFRICA

Private Bag X138, Pretoria, 0001
Delpen Building, c/o Annie Botha & Union Street, Riviera, 0084

From: Directorate Animal Health
Tel: 012 319 7532
Fax: 012 319 7470
E-mail: HerryG@daff.gov.za
Enquiries: Mr. Herry Gololo
Our Ref: 12/11/1/1/13
Your Ref No : Appendix 6

Prof Janusz Paweska and Dr Wanda Markotter
NICD and University of Pretoria

Dear Prof Paweska and Dr. Markotter

RE: Permission to do research in terms of Section 20 of the ANIMAL DISEASES ACT, 1984 (ACT NO. 35 of 1984)

Your fax / memo / letter/ Email dated 22 October 2012, requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers.

I am pleased to inform you that permission is hereby granted to perform the following research/study, with the following conditions :

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;

2. The entire research project must be conducted at the BSL4 facility at the CEZD, NICD;

3. No samples may be removed from this BSL4 facility at the NICD;

4. Solid Waste Technologies SA must be used as waste removal company

Title of research/study: Experimental infection of *Rousettus aegyptiacus* and *Epomophorus* spp wutg filoviruses

Researcher (s): Prof Paweska and Dr Wanda Markotter

Institution: NICD

Your Ref./ Project Number: Appendix 6

Our ref Number: 12/11/1/1/13

Kind regards

for **DIRECTOR: ANIMAL HEALTH**

Date:

2012 -12- 20

DEPARTMENT OF ECONOMIC DEVELOPMENT, ENVIRONMENT & TOURISM: LIMPOPO PROVINCE
DIRECTORATE: WILDLIFE TRADE AND REGULATION PERMIT

(Date Stamp) CITES & PERMIT MANAGEMENT ENVIRONMENTAL AFFAIRS LIMPOPO PROVINCE

[Issued in terms of the provisions of the Limpopo Environmental Management Act, 2003 (Act No. 7 of 2003)

CPM 006806

Receipt no. 1020399
Value R 50-00

Name of residential address of permit / licence holder: DR. WANDA MARKOTTER DEPT OF MICROBIOLOGY and Plant Pathology UNIVERSITY OF PRETORIA

Name of Land Owner ____________

In terms of and subject to the provisions of the Limpopo Environmental Management Act, 2003 (Act No. 7 of 2003) and the regulations contained therein, the holder of this permit / licence is hereby authorized to: DO SCIENTIFIC RESEARCH ON MAMMALS

during the period of validity of this permit, and subject to the conditions and requirements of this permit.

SPECIES		SEX	NUMBER	TUSK/HORN No./ MICROCHIP No./ SAFRING No.	DESCRIPTION / DERIVATIVE
COMMON NAME	SCIENTIFIC NAME				
EGYPTIAN FRUIT BAT	Rousettus aegytiacus				RESEARCH: DISEASE ECOLOGY OF
Long fingered insectivorous bat	miniopterus species				ZOONOTIC VIRUSES ASSOCIATED WITH
					ROUSETTUS AEGYTIACUS EGYPTIAN
					FRUIT BAT AND MINIOPTERUS SPECIES
					IN LIMPOPO, SOUTH AFRICA

*DELETE IF NOT APPLIICABLE

PROPERTY	*ON	*FROM	*TO
NAME AND No.	[JOHN GREAVES] LOUIS TRICHARDT ARGA, Soutpansberg,	Ornies cave in Makefe,	LEKGALAMEETSE N/R,
DISTRICT	VHEMBE, CAPRICORN AND MOPANI,		
LOCAL MUNICIPALITY	/		
PROVINCE	LIMPOPO		
COUNTRY	R.S.A		

Period of validity: From 03 / 05 / 2012 To 03 / 05 / 2017

For: Member of Executive Council, Economic Development, Environment and Tourism

T. T. SEAKAMELA
Print name

Signature of Permit Holder

FIND ATTACHED TO THIS PERMIT THE NUMBER OF ____________ SPECIAL CONDITIONS

CPB6 003767

PREMIER OF THE PROVINCE OF GAUTENG

DIRECTORATE: NATURE CONSERVATION

PERMIT TO IMPORT INTO THE PROVINCE A LIVE WILD ANIMAL

(Issued in terms of the provisions of the Nature Conservation Ordinance 12 of 1983)

GAUTENG PROVINCIAL GOVERNMENT
DEPARTMENT OF AGRICULTURE AND RURAL DEVELOPMENT
2012 -05- 25
DIRECTORATE OF CONSERVATION
PERMITS OFFICE
PO BOX 8769 JOHANNESBURG 2000

PARTICULARS OF THE PERMIT

Name and residential or business address of holder of permit:

Dr Wanda Markotter, University of Pretoria
Lunnwood Road, Dept. of Microbiology & Plant Pathology, Faculty of Natural & Agricultural Sciences, Agricultural Building
R 9-2

DETAILS OF PERMIT

FROM	DETAILS OF IMPORT	DESTINATION	ACTUALLY RECEIVED
Ornie's Cave in Mafefe at the back of Lekgalameetse Nature Reserve & Sinkhole on Lekgalameetse, Limpopo	20 (Twenty) Egyptian Fruit Bat (Rousettus aegyptiacus)		
	20 (Twenty) Long fingered insectivorous Bat (Miniopterus spp.)		
	160 (One Hundred & Sixty) Egyptian Fruit Bat (Rousettus aegyptiacus)		
	40 (Forty) Long Fingered insectivorous Bat (Miniopterus spp.)		
	Conditions: Subject to a ~~vld~~ valid export permit from Limpopo Province		
	Conditions attached		

Date and signature of permit holder to be appended here .....

In terms of and subject to the provisions of the Nature Conservation Ordinance, 1983 (Ordinance 12 of 1983) and the regulations framed thereunder, the above-mentioned person is hereby authorized, subject to the conditions appearing on this permit, to import into the Province the live wild animal/s, referred to above from the place referred to above during the period of validity of this permit and to convey it by the shortest route to the destination referred to above.

Period of validity of permit: From the date of issue to 24-11-2012

.....
FOR PREMIER

.....
Signature of holder of permit

(See conditions on reverse side)

V&R Printing Works (012) 328 7180 (11690)

Permit Number: CPB6-003767 **Permit Office Ref:** 1018

Permit Holder: Dr. Wanda Markotter

University of Pretoria

Lynnwood Road

Dept. of Microbiology & Plant

Pathology, Faculty of Natural &

Agricultural Sciences

Agricultural Building

R9-2

CONDITION TO IMPORT LIVE BAT SPECIMENS FROM LIMPOPO

- The permit is valid for six (6) months and allows import from Limpopo to Gauteng
- The animals must be transported and kept in appropriate enclosures that will ensure comfort of the animals and accommodate salient features of their ecology, physiology and behaviour
- Care must be taken to ensure minimal harm to the animals
- All dead specimens must be logged as voucher specimens with a recognised Natural History Museum

For Premier

Signature of Permit Holder

----------/----------/----------

Date

GAUTENG PROVINCIAL GOVERNMENT
DEPARTMENT OF AGRICULTURE AND RURAL DEVELOPMENT
2012 -05- 25
DIRECTORATE OF CONSERVATION
PERMITS OFFICE
PO BOX 8769 JOHANNESBURG 2000

APPENDIX B – BATS USED FOR THE DEVELOPMENT AND EVALUATION OF SEROLOGICAL ASSAYS

PANEL 1

Description: Control bat sera. The positive control was derived from sera pooled from bats experimentally infected with Marburg virus (Paweska *et al*., 2015). The negative control was derived from sera pooled from six juvenile bats born in captivity. These sera were also used for assay optimisation.

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]
Various	Various	Various	100.0	100.0
Various	Juvenile	Various	7.7	9.1

Abbreviations: ID – identification number; MARV – Marburg virus; GP – glycoprotein; NP – nucleoprotein; ELISA – enzyme-linked immunosorbent assay; PP – percent positivity
Positive results are indicated in red.
[a] Cut-off for MARV GP ELISA – 17.1 PP
[b] Cut-off for MARV NP ELISA – 25.7 PP

PANEL 2

Description: Bats experimentally infected with Ebola during a previous experiment (Paweska *et al*., 2016). Used to determine the analytical and diagnostic specificity of each I-ELISA**.**

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]	Commercial EBOV GP ELISA result (PP)[d]	DPI	PCR result
p1	Sub-adult	Female	6.1	27.1	3.7	179.0	28	Neg
p31	Sub-adult	Female	2.4	13.2	4.4	133.0	37	Neg
p104	Juvenile	Female	5.9	31.6	6.8	173.9	37	Neg
p112	Juvenile	Female	1.8	18.8	3.2	87.9	10	Neg
p113	Juvenile	Female	5.6	30.2	6.6	169.0	21	Neg

Abbreviations: ID – identification number; MARV – Marburg virus; GP – glycoprotein; NP – nucleoprotein; ELISA – enzyme-linked immunosorbent assay; PP – percent positivity; EBOV – Ebola virus; DPI – days post inoculation; PCR – polymerase chain reaction; Neg- negative
Positive results are indicated in red.
[a] Cut-off for MARV GP ELISA – 17.1 PP [b] Cut-off for MARV NP ELISA – 25.7 PP [c] Cut-off for commercial MARV GP ELISA – 16.8 PP [d] Cut-off for EBOV GP ELISA – 36.7 PP

PANEL 3
Description: Juvenile bats with no residual maternal immunity. Brought into captivity at approximately 2 months of age. Used to determine assay cut-off values and diagnostic specificity, and for assay comparison.

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]	PCR result
2016/01	Juvenile	Male	0.2	1.1	0.9	Neg
2016/02	Juvenile	Female	0.1	12.6	1.8	Neg
2016/03	Juvenile	Female	2.6	2.3	2.4	Neg
2016/04	Juvenile	Female	1.5	11.2	2.2	Neg
2016/05	Juvenile	Male	0.7	1.3	1.1	Neg
2016/06	Juvenile	Male	8.2	7.6	5.1	Neg
2016/08	Juvenile	Male	1.5	0.5	1.1	Neg
2016/12	Juvenile	Female	3.6	8.9	4.1	Neg
2016/13	Juvenile	Male	1.2	2.3	1.1	Neg
2016/14	Juvenile	Male	3.5	1.2	3.0	Neg
2016/15	Juvenile	Male	1.9	3.5	2.3	Neg
2016/17	Juvenile	Female	1.1	2.2	1.4	Neg
2016/18	Juvenile	Female	0.9	2.4	1.8	Neg
2016/21	Juvenile	Male	1.9	2.1	2.0	Neg
2016/22	Juvenile	Male	1.7	6.5	3.1	Neg
2016/23	Juvenile	Male	1.4	2.3	1.8	Neg
2016/24	Juvenile	Female	0.2	2.8	0.9	Neg
2016/26	Juvenile	Female	4.1	7.0	5.3	Neg
2016/27	Juvenile	Female	2.0	0.9	2.5	Neg
2016/29	Juvenile	Female	0.1	2.6	0.8	Neg
2016/31	Juvenile	Female	9.8	9.9	8.9	Neg
2016/32	Juvenile	Female	13.1	10.2	11.6	Neg
2016/33	Juvenile	Male	1.4	2.0	1.5	Neg
2016/34	Juvenile	Male	1.7	0.7	1.5	Neg
2016/35	Juvenile	Female	0.8	0.6	1.3	Neg

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]	PCR result
SMB589	Juvenile	Male	0.1	2.5	1.6	Neg

Abbreviations: ID – identification number; MARV – Marburg virus; GP – glycoprotein; NP – nucleoprotein; ELISA – enzyme-linked immunosorbent assay; PP – percent positivity; EBOV – Ebola virus; DPI – days post inoculation; PCR – polymerase chain reaction; Neg - negative

[a] Cut-off for MARV GP ELISA – 17.1 PP

[b] Cut-off for MARV NP ELISA – 25.7 PP

[c] Cut-off for commercial MARV GP ELISA – 16.8 PP

PANEL 4

Description: Bats experimentally infected with Marburg virus (Paweska *et al*., 2015; this thesis chapter 3). Used for assay comparison and to determine diagnostic sensitivity.

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]	DPI	PCR result	VI result
6-04	Adult	Male	22.4	27.9	18.6	7	Pos	Pos
6-06	Adult	Female	148.2	145.6	146.6	9		
6-06			123.6	154.6	138.6	14		
6-06			114.8	150.0	109.4	21		
6-06			106.1	152.1	105.8	28		
6-06			48.6	144.3	56.9	42		
6-11	Adult	Female	31.9	25.7	27.0	7	Pos	Pos
6-16	Adult	Female	21.4	26.1	18.6	7	Pos	Pos
6-18	Adult	Male	22.7	63.6	17.0	9	Pos	Neg
6-18			52.1	154.3	21.6	14		
6-18			47.5	168.3	38.7	21		
6-18			58.5	171.1	55.8	28		
6-18			43.8	162.6	53.1	42		
6-22	Adult	Male	97.4	96.1	122.0	9	Pos	Pos
6-24	Adult	Male	31.3	46.8	34.8	9	Pos	Pos
6-24			35.9	47.1	39.8	21		

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]	DPI	PCR result	VI result
6-24			41.7	49.5	57.6	28		
6-25	Adult	Female	17.6	26.1	18.9	5	Pos	Pos
6-26	Adult	Female	147.1	6.7	174.8	5	Pos	Neg
6-26			153.5	134.6	165.7	9		
6-26			172.8	135.9	175.9	12		
6-28	Adult	Male	84.7	150.4	86.9	9	Pos	Pos
6-28			101.6	166.6	166.9	12		
6-29	Adult	Male	69.5	53.2	62.6	9	Pos	Pos
6-29			40.8	165.2	75.1	14		
6-31	Adult	Male	18.9	21.9	17.8	5	Pos	Pos
6-32	Adult	Male	18.1	26.2	17.4	5	Pos	Pos
6-34	Adult	Male	155.9	168.7	148.2	9	Pos	Neg
6-36	Adult	Female	25.1	35.7	24.2	9	Pos	Pos
6-36			26.2	42.1	23.8	12		
6-37	Adult	Female	17.3	113.7	57.1	9	Pos	Neg
6-37			52.7	152.2	71.4	21		
6-37			41.9	148.7	54.8	28		
6-37			46.3	148.6	41.9	42		
6-41	Adult	Female	31.9	42.1	28.4	9	Pos	Neg
6-41			40.1	65.7	37.4	14		
SMB458	Adult	Female	33.2	146.9	54.9	14	Pos	Neg
SMB458			34.8	142.8	58.9	21		
SMB458			33.8	127.7	51.3	28		
SMB458			19.2	79.7	25.6	42		
SMB458			19.3	55.1	22.9	67		
SMB458			19.3	48.2	22.4	89		
SMB458			15.8	37.2	22.1	110		

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]	DPI	PCR result	VI result
SMB499	Adult	Female	131.6	147.0	129.5	14	Pos	Neg
SMB499			129.9	146.5	129.1	21		
SMB499			126.8	151.1	122.7	28		
SMB499			109.5	143.2	115.7	42		
SMB499			77.4	130.1	98.3	67		
SMB499			72.5	124.1	91.4	89		
SMB499			45.7	117.7	53.8	110		
SMB577	Juvenile	Male	32.6	108.4	41.5	14	Pos	Neg
SMB577			26.1	64.8	39.3	21		
SMB577			22.1	58.2	31.9	28		
SMB577			18.8	52.1	30.1	42		
SMB577			18.1	44.6	28.7	67		
SMB577			17.7	34.9	28.7	89		
SMB577			17.3	27.2	26.4	110		
12-04-16	Juvenile	Female	74.7	53.7	81.3	9	Pos	Pos
12-04-16			130.1	147.1	91.9	14		
12-04-16			125.0	139.5	126.4	21		
12-04-16			124.5	137.0	127.8	28		
12-04-16			104.7	124.4	110.2	42		
12-04-16			70.9	103.1	65.1	67		
12-04-16			62.1	88.7	63.1	89		
12-04-16			56.4	78.4	59.8	110		
16/04/03	Juvenile	Male	105.1	52.7	98.4	9	Pos	Pos
16/04/03			117.4	107.4	102.3	14		
16/04/03			106.2	84.8	101.7	21		
16/04/03			105.7	82.1	106.8	28		
16/04/03			90.7	54.7	89.5	42		

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]	DPI	PCR result	VI result
16/04/03			87.1	26.9	85.4	67		
16/04/03			72.5	25.7	79.9	89		
16/04/03			60.0	16.1	76.4	110		
2016/06	Juvenile	Male	27.1	97.8	25.9	9	Pos	Pos
2016/06			56.2	152.4	67.3	14		
2016/06			52.4	150.0	64.2	21		
2016/06			49.2	147.3	55.9	28		
2016/06			48.6	144.3	50.7	42		
2016/06			28.5	140.2	33.3	67		
2016/06			26.7	135.4	31.3	89		
2016/06			19.2	123.6	28.1	110		

Abbreviations: ID – identification number; MARV – Marburg virus; GP – glycoprotein; NP – nucleoprotein; ELISA – enzyme-linked immunosorbent assay; PP – percent positivity; DPI – days post inoculation; PCR – polymerase chain reaction; VI – virus isolation; Neg – negative; Pos - positive
Positive results are indicated in red.
[a] Cut-off for MARV GP ELISA – 17.1 PP
[b] Cut-off for MARV NP ELISA – 25.7 PP
[c] Cut-off for commercial MARV GP ELISA – 16.8 PP

PANEL 5
Description: Bats that served as controls during a Marburg virus experimental infection study (Paweska *et al*., 2015; this thesis chapter 3). Includes day 0 post-infection sera from experimentally infected bats. Used for assay comparison and to determine diagnostic specificity.

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]	DPI (Mock inoculated)	PCR
6-06	Adult	Female	0.1	6.0	4.9	0	Neg
6-08	Adult	Male	8.8	11.5	9.4		Neg
6-08			9.1	10.9	10.2		Neg

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]	DPI (Mock inoculated)	PCR
6-08			9.5	11.1	10.1		Neg
6-08			9.4	10.8	9.5		Neg
6-10	Adult	Female	8.8	11.9	8.4		Neg
6-10			8.6	11.8	8.7		Neg
6-10			8.6	12.1	8.1		Neg
6-10			8.7	11.5	7.0		Neg
6-14	Adult	Female	8.4	10.1	7.3		Neg
6-14			8.1	9.6	7.7		Neg
6-14			7.6	10.1	7.4		Neg
6-14			7.7	10.3	6.7		Neg
6-17	Adult	Male	8.8	14.2	9.1		Neg
6-17			8.4	15.1	8.9		Neg
6-17			8.4	14.8	8.7		Neg
6-17			8.5	14.7	9.5		Neg
6-18	Adult	Male	2.6	3.8	6.1	0	Neg
6-19	Adult	Female	10.5	14.2	8.5		Neg
6-19			10.1	14.3	10.2		Neg
6-19			10.4	14.9	9.6		Neg
6-19			10.4	14.2	8.9		Neg
6-21	Adult	Male	5.2	8.2	7.1		Neg
6-21			5.6	8.2	7.4		Neg
6-21			5.4	8.1	7.2		Neg
6-21			5.4	8.2	7.3		Neg
6-22	Adult	Male	0.3	2.9	10.6	0	Neg
6-26	Adult	Female	12.1	14.5	13.8	0	Neg
6-29	Adult	Male	0.6	6.4	5.4	0	Neg
6-30	Adult	Male	9.9	8.2	11.0		Neg

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]	DPI (Mock inoculated)	PCR
6-30			9.5	8.7	11.1		Neg
6-30			9.8	8.7	10.7		Neg
6-30			9.6	8.4	8.2		Neg
6-33	Adult	Female	8.4	10.9	9.1		Neg
6-33			9.1	13.4	8.2		Neg
6-33			9.3	13.1	8.8		Neg
6-33			9.2	13.1	10.1		Neg
6-34	Adult	Male	8.3	7.7	11.6	0	Neg
6-37	Adult	Female	3.0	5.9	4.6	0	Neg
6-38	Adult	Male	5.1	8.2	7.8		Neg
6-38			6.9	11.5	7.1		Neg
6-38			7.1	10.4	7.4		Neg
6-38			7.2	8.3	7.4		Neg
6-39	Adult	Male	8.1	12.8	9.0		Neg
6-39			7.3	9.7	7.8		Neg
6-39			8.1	14.8	8.2		Neg
6-39			7.2	6.2	7.6		Neg
6-40	Adult	Male	8.2	9.9	8.7		Neg
6-40			10.6	8.3	9.1		Neg
6-40			5.7	12.5	8.8		Neg
6-40			8.6	8.1	7.9		Neg
6-44	Adult	Female	7.1	11.2	7.9		Neg
6-44			8.5	9.6	8.0		Neg
6-44			7.9	11.3	8.2		Neg
6-44			8.9	10.2	7.0		Neg
6-46	Adult	Female	14.2	18.3	16.1		Neg
6-46			13.7	18.1	16.5		Neg

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]	DPI (Mock inoculated)	PCR
6-46			13.7	19.0	16.4		Neg
6-46			13.5	18.2	14.8		Neg
SMB458	Adult	Female	6.8	7.6	6.6	0	Neg
SMB499	Adult	Female	12.5	11.2	10.8	0	Neg
SMB577	Juvenile	Male	9.5	8.7	6.3	0	Neg
12-04-16	Juvenile	Female	16.2	11.4	15.4	0	Neg
16/04/03	Juvenile	Male	12.5	7.2	9.9	0	Neg
2016/06	Juvenile	Male	8.2	7.6	6.1	0	Neg

Abbreviations: ID – identification number; MARV – Marburg virus; GP – glycoprotein; NP – nucleoprotein; ELISA – enzyme-linked immunosorbent assay; PP – percent positivity; DPI – days post inoculation; PCR – polymerase chain reaction; VI – virus isolation; Neg – negative

[a] Cut-off for MARV GP ELISA – 17.1 PP

[b] Cut-off for MARV NP ELISA – 25.7 PP

[c] Cut-off for commercial MARV GP ELISA – 16.8 PP

PANEL 6

Description: Wild-caught bats; sampled and released at Matlapitsi cave, Limpopo. Previously tested using an I-ELISA based on commercially available recombinant MARV GP (Paweska *et al*., 2018). Used for assay comparison.

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
5174	Sub-adult	Male	15.3	19.7	14.8
5175	Sub-adult	Female	9.6	7.4	7.7
5176	Sub-adult	Female	2.8	4.7	1.2
5177	Sub-adult	Female	1.9	10.4	1.1
5178	Sub-adult	Female	0.5	3.2	0.1
5179	Sub-adult	Female	1.1	2.9	1.2
5180	Sub-adult	Female	0.9	5.1	0.5

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
5181	Sub-adult	Male	15.4	20.1	16.6
5182	Sub-adult	Male	2.7	1.9	2.7
5190	Sub-adult	Female	10.8	11.6	9.3
5192	Sub-adult	Female	0.8	2.2	0.4
5193	Sub-adult	Male	1.4	1.9	0.9
5194	Adult	Male	103.5	146.9	120.6
5195	Sub-adult	Female	0.6	5.3	0.0
5196	Sub-adult	Male	5.9	7.7	4.3
5197	Sub-adult	Female	16.7	21.4	15.8
5198	Sub-adult	Female	1.8	3.2	2.2
5199	Sub-adult	Female	1.9	2.1	1.5
5200	Sub-adult	Male	1.9	5.1	1.9
5201	Sub-adult	Female	40.6	61.9	34.4
5202	Sub-adult	Female	0.6	2.6	0.8
5203	Sub-adult	Male	6.7	10.3	8.2
5204	Sub-adult	Male	3.8	15.2	9.3
5205	Sub-adult	Male	9.9	13.3	11.2
5207	Sub-adult	Male	1.5	4.1	0.6
5208	Sub-adult	Female	0.7	2.9	0.7
5209	Sub-adult	Female	4.7	11.2	4.4
5210	Sub-adult	Male	4.6	4.2	3.3
5211	Sub-adult	Female	5.9	9.1	6.7
5212	Sub-adult	Female	15.2	19.4	12.9
5213	Sub-adult	Female	1.6	2.8	3.3
5214	Sub-adult	Female	1.1	5.2	0.2
5215	Adult	Male	4.7	7.3	4.3
5216	Adult	Male	56.8	64.9	33.7

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
5217	Adult	Male	100.3	106.7	86.4
5218	Sub-adult	Male	4.2	8.2	5.1
5219	Adult	Female	40.6	67.1	41.1
5220	Sub-adult	Female	15.2	16.9	13.2
5221	Sub-adult	Male	0.9	2.5	0.1
5222	Sub-adult	Male	9.1	15.3	10.0
5223	Sub-adult	Female	17.8	23.2	15.3
5224	Adult	Female	8.2	9.5	8.3
5225	Sub-adult	Male	16.1	18.3	14.6
5226	Sub-adult	Male	0.9	1.1	0.2
5227	Adult	Male	30.2	42.9	28.8
5228	Sub-adult	Male	36.7	89.3	41.5
5229	Sub-adult	Female	17.9	26.8	19.4
5230	Adult	Male	11.9	16.3	10.6
5239	Sub-adult	Female	0.9	5.2	0.8
5240	Sub-adult	Male	9.7	3.5	8.3
5241	Sub-adult	Female	0.8	3.7	0.1
5242	Sub-adult	Female	0.3	4.9	0.2
5243	Sub-adult	Female	6.8	0.5	5.3
5244	Sub-adult	Male	1.1	2.9	0.8
5245	Sub-adult	Female	1.3	1.9	1.9
5246	Sub-adult	Female	1.5	2.3	0.4
5247	Adult	Male	35.1	59.7	43.2
5248	Sub-adult	Male	1.6	3.4	0.8
5249	Sub-adult	Female	1.9	5.1	1.0
5250	Sub-adult	Female	2.9	9.3	3.2
5251	Sub-adult	Male	2.1	10.9	1.4

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
5252	Sub-adult	Male	8.7	14.8	9.4
5375	Adult	Male	9.2	17.5	10.0
5376	Adult	Male	0.7	3.7	0.1
5377	Adult	Female	86.9	42.6	71.5
5378	Adult	Female	2.5	0.9	0.6
5382	Sub-adult	Male	80.1	78.2	64.2
5383	Sub-adult	Female	3.6	5.9	0.6
5384	Sub-adult	Female	5.3	6.1	4.4
5385	Adult	Female	4.1	9.3	3.5
5386	Sub-adult	Male	2.6	7.6	1.6
5387	Adult	Male	5.5	12.8	3.2
5388	Adult	Female	12.6	19.9	15.3
5389	Sub-adult	Male	1.1	4.2	1.5
5390	Sub-adult	Male	4.4	13.1	5.1
5391	Adult	Male	0.9	1.8	0.6
5392	Sub-adult	Male	6.2	11.3	7.1
5393	Sub-adult	Female	4.4	7.2	3.6
5394	Sub-adult	Female	65.9	94.2	63.1
5404	Sub-adult	Female	1.1	3.5	0.3
5405	Adult	Female	1.3	4.2	0.4
5406	Adult	Male	95.4	32.6	103.4
5407	Adult	Male	70.1	111.3	77.4
5408	Adult	Male	89.3	36.9	102.0
5409	Sub-adult	Female	1.2	5.6	0.7
5410	Juvenile	Female	1.6	1.9	0.4
5411	Sub-adult	Male	2.7	1.1	0.4
5412	Adult	Male	110.7	63.5	122.8

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
5413	Adult	Female	16.5	21.7	16.0
5414	Sub-adult	Female	4.1	9.6	0.2
5415	Sub-adult	Female	2.8	7.1	3.6
5416	Adult	Female	140.4	101.3	134.9
5417	Sub-adult	Male	1.3	3.8	0.7
5418	Sub-adult	Male	3.5	9.9	2.9
5419	Sub-adult	Female	6.2	8.9	6.0
5420	Sub-adult	Male	1.8	13.7	0.9
5421	Adult	Female	5.9	5.1	4.4
5422	Sub-adult	Male	20.5	55.6	29.1
5423	Adult	Female	58.9	88.1	61.2
5424	Sub-adult	Male	1.7	3.3	2.0
5425	Adult	Male	129.2	88.3	142.2
5426	Sub-adult	Female	170.1	151.7	175.4
5427	Adult	Male	155.3	180.4	176.4
5428	Sub-adult	Male	1.1	3.4	1.9
5434	Adult	Female	1.2	5.7	0.8
5435	Adult	Male	68.1	99.8	59.7
5436	Adult	Female	16.5	21.2	14.8
5437	Sub-adult	Female	10.8	10.4	6.5
5438	Adult	Male	1.9	4.1	0.3
5439	Adult	Male	163.4	120.1	175.3
5440	Sub-adult	Male	2.2	4.1	1.7
5441	Sub-adult	Female	1.3	1.5	0.6
5442	Sub-adult	Female	2.1	6.8	1.4
5443	Adult	Male	76.8	45.9	77.5
5444	Sub-adult	Female	2.4	7.5	0.8

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
5445	Sub-adult	Female	8.9	3.6	10.4
5446	Sub-adult	Female	38.9	67.7	43.5
5447	Sub-adult	Male	1.2	3.2	1.1
5448	Adult	Male	17.6	25.9	18.3
5609	Sub-adult	Female	11.3	20.1	13.9
5610	Sub-adult	Female	21.8	35.9	17.3
5611	Sub-adult	Female	56.3	22.4	49.2
5613	Sub-adult	Female	41.1	63.8	35.8
5614	Sub-adult	Female	127.4	155.2	131.9
5615	Adult	Male	18.1	38.1	17.6
5616	Sub-adult	Female	11.7	15.2	13.2
5617	Sub-adult	Female	45.5	51.3	43.2
5620	Sub-adult	Female	65.2	81.6	71.5
5621	Sub-adult	Female	145.4	98.5	189.1
5622	Adult	Male	110.1	153.8	106.1
5623	Sub-adult	Female	25.3	42.6	33.4
5624	Sub-adult	Female	60.9	82.4	65.8
5626	Sub-adult	Female	75.4	113.7	90.3
5627	Sub-adult	Female	4.4	7.2	3.7
5628	Sub-adult	Female	89.7	62.4	84.1
5629	Sub-adult	Female	1.4	3.2	0.7
5630	Sub-adult	Female	51.1	73.4	46.7
5631	Adult	Female	16.5	23.8	15.9
5632	Sub-adult	Female	98.8	123.6	105.1
5633	Sub-adult	Female	25.9	40.2	23.2
5634	Sub-adult	Female	79.9	117.5	81.5
5635	Sub-adult	Male	8.2	21.3	7.1

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
5636	Sub-adult	Female	21.1	29.6	18.5
5637	Adult	Female	72.4	88.7	63.8
5638	Sub-adult	Female	69.2	46.3	71.1
5639	Sub-adult	Female	10.1	26.5	9.7
5640	Sub-adult	Male	168.8	79.4	165.7
5641	Adult	Male	88.9	76.2	91.2
5642	Sub-adult	Female	22.4	29.7	23.7
5647	Sub-adult	Female	155.1	104.6	132.1
5648	Sub-adult	Male	27.3	45.5	29.8
5649	Sub-adult	Female	1.9	2.4	1.6
5650	Sub-adult	Female	103.6	129.7	111.3
5651	Sub-adult	Female	29.9	54.2	32.8
5652	Sub-adult	Male	43.7	28.9	65.5
5653	Sub-adult	Male	162.4	153.2	171.7
5654	Sub-adult	Female	2.2	6.2	4.6
5655	Sub-adult	Male	21.1	73.4	24.7
5656	Sub-adult	Female	144.2	97.3	136.8
5657	Sub-adult	Male	1.3	5.2	0.3
5658	Sub-adult	Female	18.7	37.5	17.9
5659	Sub-adult	Male	153.4	64.2	124.2
5660	Sub-adult	Male	19.8	42.1	16.9
5661	Adult	Male	66.2	62.7	61.2
5662	Sub-adult	Male	157.3	89.5	147.1
5663	Sub-adult	Female	65.2	91.3	60.9
5664	Sub-adult	Male	16.5	24.3	15.9
5665	Sub-adult	Male	57.4	77.9	60.3
5666	Sub-adult	Male	50.1	25.8	48.9

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
5667	Sub-adult	Male	1.4	6.2	2.4
5668	Sub-adult	Male	4.2	7.1	4.0
5669	Sub-adult	Male	61.3	74.2	67.0
5670	Sub-adult	Male	25.5	58.9	26.0
5671	Sub-adult	Male	22.4	35.3	29.4
5672	Sub-adult	Male	47.2	26.1	40.6
5673	Sub-adult	Male	4.9	7.8	3.6
5674	Sub-adult	Male	74.8	44.2	69.4
5675	Sub-adult	Male	65.3	83.6	64.5
5676	Sub-adult	Female	4.1	9.9	5.4
5677	Adult	Male	21.0	33.3	21.4
5678	Sub-adult	Male	160.4	84.5	158.2
5679	Sub-adult	Female	91.7	66.5	95.8
5680	Sub-adult	Female	16.8	21.8	15.2
5681	Sub-adult	Female	65.1	111.6	84.6
5682	Sub-adult	Male	15.9	25.3	16.0
5683	Sub-adult	Male	1.9	5.2	2.6
5847	Sub-adult	Female	41.1	67.8	45.5
5848	Sub-adult	Female	15.6	19.5	14.1
5849	Sub-adult	Female	52.8	88.3	45.2
5850	Sub-adult	Female	89.9	26.8	97.6
5851	Adult	Female	61.2	53.7	61.8
5852	Adult	Female	28.9	26.0	37.8
5853	Adult	Male	10.7	22.5	11.3
5854	Sub-adult	Male	25.8	28.6	28.4
5855	Adult	Female	75.1	30.4	69.7
5856	Sub-adult	Female	124.3	99.8	101.5

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
5857	Sub-adult	Female	3.3	6.1	2.7
5858	Adult	Female	96.4	77.3	98.1
5859	Sub-adult	Female	17.8	45.2	18.9
5860	Sub-adult	Male	14.9	21.8	15.3
5861	Sub-adult	Female	20.2	26.1	25.5
5863	Sub-adult	Female	89.9	65.3	91.0
5864	Adult	Male	21.5	42.6	20.0
5865	Adult	Male	58.1	52.8	52.5
5866	Adult	Male	11.7	16.5	14.3
5867	Sub-adult	Female	29.6	43.1	33.8
5868	Adult	Female	68.8	25.8	73.8
5869	Sub-adult	Female	6.9	9.4	7.1
5870	Adult	Female	1.5	7.3	0.7
5871	Adult	Female	140.6	165.9	139.2
5872	Adult	Female	35.8	37.3	37.9
5873	Sub-adult	Male	20.5	35.2	19.6
5874	Adult	Female	67.3	92.7	50.1
5875	Adult	Female	126.4	103.6	112.7
5876	Adult	Male	100.7	131.8	96.6
5877	Sub-adult	Male	102.9	155.5	113.2
5878	Adult	Female	52.4	26.9	49.5
5879	Adult	Female	59.2	68.4	51.4
5880	Adult	Female	60.8	60.2	56.5
6123	Juvenile	Female	55.3	30.4	54.3
6124	Adult	Female	21.9	20.0	17.6
6126	Sub-adult	Male	99.6	39.0	113.4
6127	Sub-adult	Male	31.6	46.3	39.2

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
6129	Juvenile	Male	48.8	25.0	41.8
6131	Adult	Female	13.0	9.4	5.8
6133	Juvenile	Female	55.8	27.2	58.9
6135	Adult	Female	14.6	13.9	10.8
6137	Juvenile	Male	17.5	25.2	10.0
6145	Sub-adult	Female	34.2	16.6	52.6
6146	Sub-adult	Female	4.1	3.9	14.9
6148	Sub-adult	Female	9.7	18.4	11.2
6149	Juvenile	Male	28.3	25.8	29.6
6150	Juvenile	Male	21.8	32.2	26.9
6151	Juvenile	Male	12.7	31.0	9.4
6153	Adult	Female	33.9	32.5	35.3
6154	Adult	Female	23.6	29.7	23.0
6155	Adult	Female	25.7	27.2	22.2
6156	Juvenile	Female	11.5	8.1	8.2
6157	Adult	Female	6.9	8.5	8.3
6158	Adult	Female	46.0	20.2	30.3
6159	Juvenile	Male	39.1	29.2	33.2
6161	Adult	Female	15.5	47.1	7.1
6162	Adult	Female	26.0	27.0	19.8
6168	Sub-adult	Male	38.1	141.3	54.6
6169	Adult	Female	61.9	46.8	57.6
6170	Adult	Female	92.6	26.6	106.8
6171	Adult	Female	11.7	5.4	8.3
6172	Adult	Female	43.4	39.3	41.3
6173	Adult	Female	9.2	8.9	4.2
6174	Adult	Female	24.7	21.4	17.7

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
6175	Adult	Female	33.5	27.9	28.7
6176	Adult	Female	24.8	41.0	47.1
6177	Adult	Male	28.9	79.7	26.9
6178	Adult	Female	22.7	26.6	16.9
6179	Adult	Female	16.6	16.2	16.5
6180	Adult	Female	23.6	28.0	14.2
6181	Adult	Female	23.0	25.9	17.4
6182	Adult	Female	59.8	33.3	54.6
6183	Adult	Female	127.9	26.8	124.2
6184	Adult	Female	58.1	23.3	65.1
6185	Sub-adult	Female	8.2	11.9	0.7
6186	Sub-adult	Female	14.7	18.0	7.7
6187	Sub-adult	Female	33.8	33.4	38.6
6188	Sub-adult	Female	19.6	65.7	30.6
6189	Adult	Male	16.2	24.0	12.9
6190	Juvenile	Female	16.9	22.0	14.3
6191	Sub-adult	Female	36.5	27.2	40.3
6192	Sub-adult	Female	59.2	32.2	70.3
6193	Adult	Female	48.4	106.8	42.5
6194	Adult	Female	11.2	17.0	8.7
6195	Sub-adult	Female	46.1	91.6	92.7
6196	Adult	Female	16.4	23.2	13.0
6197	Adult	Female	21.9	27.2	17.1
6198	Adult	Female	16.1	17.5	14.7
6199	Adult	Female	82.1	29.9	87.0
6201	Adult	Female	32.4	30.2	29.9
6202	Sub-adult	Female	12.7	24.7	6.7

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
6203	Sub-adult	Female	17.2	34.4	18.1
6204	Adult	Female	8.9	25.1	15.4
6205	Sub-adult	Female	38.3	47.5	36.4
6206	Adult	Female	25.8	97.1	27.9
6207	Sub-adult	Female	15.5	15.1	8.4
6208	Adult	Male	38.1	44.0	41.4
6209	Sub-adult	Female	50.8	29.0	41.1
6419	Sub-adult	Female	29.0	4.7	25.4
6420	Juvenile	Female	5.4	8.8	1.6
6421	Juvenile	Female	1.4	6.5	1.5
6422	Sub-adult	Female	3.9	6.7	2.4
6423	Juvenile	Male	0.9	18.1	0.4
6424	Sub-adult	Female	17.9	26.9	16.9
6425	Juvenile	Male	8.1	4.4	6.1
6426	Juvenile	Male	2.6	10.1	1.9
6427	Juvenile	Female	5.8	4.8	2.3
6428	Juvenile	Female	4.0	9.5	4.4
6429	Juvenile	Female	7.0	7.5	5.2
6430	Sub-adult	Female	1.6	7.0	0.6
6431	Juvenile	Female	3.0	5.4	2.6
6432	Juvenile	Male	18.5	27.5	19.9
6433	Juvenile	Female	16.4	12.0	13.2
6434	Juvenile	Female	2.2	5.2	0.9
6435	Juvenile	Female	2.0	5.8	2.2
6436	Juvenile	Female	4.1	10.4	2.2
6437	Juvenile	Male	3.5	8.5	2.4
6438	Sub-adult	Male	2.4	7.4	0.4

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
6439	Juvenile	Male	11.0	13.1	11.6
6440	Sub-adult	Male	9.3	8.9	5.2
6441	Juvenile	Male	3.4	7.8	2.6
6442	Sub-adult	Male	6.1	12.5	4.1
6443	Sub-adult	Male	4.0	6.0	3.4
6444	Sub-adult	Male	5.1	9.8	2.9
6445	Juvenile	Male	1.8	5.5	0.5
6446	Adult	Male	30.6	48.7	26.2
6447	Juvenile	Female	5.8	5.9	1.7
6448	Sub-adult	Male	1.0	18.5	2.1
6449	Juvenile	Female	5.7	7.9	3.4
6450	Juvenile	Female	1.1	6.8	0.9
6451	Juvenile	Female	6.6	7.4	6.9
6452	Juvenile	Male	3.9	6.4	3.8
6453	Sub-adult	Female	4.1	6.8	3.9
6454	Sub-adult	Female	1.8	9.4	0.2
6455	Juvenile	Male	3.3	7.2	2.7
6456	Sub-adult	Female	10.2	6.8	6.4
6457	Adult	Male	55.9	64.3	53.7
6458	Juvenile	Male	3.3	5.0	0.2
6459	Juvenile	Male	7.1	7.5	7.0
6460	Juvenile	Female	3.6	10.4	3.3
6461	Juvenile	Female	5.1	14.6	3.9
6462	Juvenile	Male	4.2	13.1	2.1
6463	Juvenile	Female	2.8	10.1	1.4
6464	Juvenile	Female	4.4	5.2	4.7
6465	Juvenile	Female	5.1	8.6	5.4

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
6466	Juvenile	Female	10.5	10.9	10.2
6467	Juvenile	Female	1.1	6.3	0.9
6468	Juvenile	Female	1.8	7.3	1.8
6469	Juvenile	Female	8.9	23.4	4.6
6470	Juvenile	Female	3.4	7.5	2.9
6471	Juvenile	Female	3.5	25.2	1.9
6472	Juvenile	Female	1.5	13.9	1.3
6473	Juvenile	Male	1.2	30.4	0.7
6474	Juvenile	Female	3.3	16.4	2.7
6475	Juvenile	Female	5.9	11.2	5.3
6476	Juvenile	Male	3.2	8.0	1.3
6478	Juvenile	Male	45.9	24.5	47.3
6479	Juvenile	Female	6.6	19.3	4.2
6480	Juvenile	Female	1.8	15.1	1.1
6481	Adult	Female	7.2	25.5	6.0
6482	Juvenile	Female	4.1	12.2	3.5
6483	Juvenile	Male	3.9	12.4	2.9
6484	Juvenile	Female	3.1	6.1	3.2
6485	Juvenile	Female	0.9	5.6	1.2
6486	Juvenile	Female	1.1	7.2	1.9
6487	Sub-adult	Male	3.6	8.0	4.0
6488	Juvenile	Female	1.5	7.7	0.4
6489	Juvenile	Female	3.8	6.9	2.7
6490	Juvenile	Male	6.2	18.4	3.3
6491	Juvenile	Female	4.7	10.6	2.2
6492	Adult	Female	2.3	12.6	1.4
6493	Juvenile	Female	7.1	9.3	2.8

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
6494	Juvenile	Female	3.1	6.5	2.9
6495	Juvenile	Female	5.9	13.4	4.1
6496	Juvenile	Male	13.6	13.1	10.8
6497	Juvenile	Female	7.7	11.6	5.1
6498	Juvenile	Female	2.9	7.4	5.4
6499	Juvenile	Female	3.3	8.1	4.1
6500	Juvenile	Female	0.9	5.8	0.2
6501	Juvenile	Male	1.8	8.2	4.6
6502	Sub-adult	Male	4.6	6.3	1.0
6503	Juvenile	Female	1.2	10.9	0.9
6504	Adult	Female	10.2	17.0	4.5
6505	Adult	Male	35.8	28.2	27.7
6506	Juvenile	Female	9.9	11.6	0.6
6508	Adult	Male	29.1	29.5	32.0
6509	Juvenile	Female	6.6	5.0	7.5
6510	Adult	Female	5.2	7.0	5.4
6511	Adult	Male	11.1	6.1	14.9
6512	Juvenile	Male	0.8	10.3	0.2
6513	Adult	Male	66.9	27.5	72.0
6514	Adult	Female	17.6	37.5	18.2
6515	Adult	Female	1.7	6.9	0.3
6516	Adult	Female	2.5	7.8	1.2
6517	Adult	Female	13.3	6.5	15.3
6844	Adult	Male	12.8	9.9	16.0
6845	Juvenile	Male	1.1	2.4	1.4
6846	Juvenile	Male	0.9	7.5	0.0
6847	Sub-adult	Male	0.7	3.9	0.4

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
6861	Sub-adult	Male	3.6	4.1	2.0
6862	Adult	Male	35.1	26.5	35.6
6863	Sub-adult	Male	5.5	10.9	2.9
6864	Sub-adult	Female	1.7	2.9	0.2
6865	Sub-adult	Female	0.3	4.3	0.1
6866	Adult	Male	7.4	18.7	9.4
6867	Sub-adult	Male	2.6	5.0	0.5
6868	Sub-adult	Male	1.9	2.1	0.2
6869	Sub-adult	Male	1.2	0.2	0.1
6870	Sub-adult	Male	33.7	49.2	35.8
6871	Sub-adult	Female	3.2	4.4	0.5
6872	Sub-adult	Female	7.2	22.0	8.3
6873	Adult	Male	9.9	5.9	13.7
6874	Sub-adult	Male	1.8	14.9	0.5
6875	Adult	Male	55.3	37.1	64.3
6876	Adult	Male	20.1	26.0	16.9
6877	Sub-adult	Male	1.3	1.5	0.5
6878	Sub-adult	Female	4.9	10.9	0.4
6879	Sub-adult	Female	0.3	3.2	0.2
6880	Sub-adult	Female	1.6	18.8	0.4
6881	Adult	Male	60.9	50.8	58.6
6882	Adult	Male	15.3	20.6	14.0
6883	Adult	Male	45.2	32.6	57.6
6884	Adult	Female	13.9	14.2	15.4
6885	Sub-adult	Female	1.4	3.4	0.2
6886	Sub-adult	Male	19.8	68.3	20.4
6887	Sub-adult	Male	11.1	13.5	12.4

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
6888	Adult	Male	5.4	24.7	3.0
6890	Adult	Male	0.3	0.9	0.2
6892	Sub-adult	Female	0.7	0.4	0.4
6893	Sub-adult	Female	20.5	27.2	19.1
6894	Sub-adult	Female	3.3	2.7	3.1
6895	Sub-adult	Female	1.9	8.7	1.1
6896	Adult	Male	35.1	28.4	36.1
6897	Juvenile	Female	33.3	95.6	28.3
6898	Sub-adult	Female	3.9	22.5	1.5
6899	Adult	Female	54.1	27.3	68.3
6900	Sub-adult	Male	2.1	4.7	0.1
6901	Sub-adult	Male	0.9	3.8	0.7
6902	Sub-adult	Male	1.6	2.1	0.8
6903	Adult	Male	22.2	25.9	21.8
6904	Adult	Male	0.7	3.4	0.1
6906	Adult	Female	31.6	110.7	27.6
6907	Adult	Female	6.1	8.6	5.4
6908	Sub-adult	Female	0.4	11.6	0.3
6909	Sub-adult	Female	0.9	8.5	0.3
6910	Sub-adult	Female	1.1	5.7	0.9
6911	Sub-adult	Female	1.6	4.9	0.8
6912	Sub-adult	Male	2.2	2.8	0.7
6913	Sub-adult	Male	0.6	2.1	0.7
6914	Sub-adult	Male	39.1	86.0	25.8
6917	Adult	Female	33.7	35.2	37.0
6918	Sub-adult	Female	7.3	13.6	0.3
6919	Adult	Female	21.6	36.8	18.5

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
6920	Adult	Male	20.5	5.4	21.4
6921	Sub-adult	Female	3.3	7.1	2.1
6922	Sub-adult	Female	9.1	23.3	6.9
6923	Sub-adult	Female	5.2	12.2	5.1
6924	Sub-adult	Male	4.3	6.3	2.2
6925	Adult	Female	22.9	34.9	25.7
7000	Adult	Female	25.6	25.9	22.0
7001	Adult	Male	14.3	19.8	15.5
7002	Adult	Female	39.9	28.1	34.8
7003	Adult	Female	46.1	55.3	39.3
7004	Adult	Female	6.2	8.9	7.5
7012	Adult	Female	17.9	33.2	18.6
7013	Adult	Female	52.7	66.8	37.3
7016	Adult	Female	29.4	51.3	31.7
7018	Adult	Female	102.5	99.4	158.3
7019	Adult	Female	19.9	27.6	20.5
7021	Adult	Male	19.3	27.1	21.1
7022	Adult	Female	187.4	155.9	161.5
7024	Adult	Female	9.8	15.6	12.5
7025	Adult	Male	3.3	12.3	4.0
7026	Adult	Female	2.2	8.7	1.9
7027	Adult	Female	10.9	17.4	12.8
7028	Adult	Male	4.4	4.5	4.5
7029	Adult	Female	66.2	96.3	61.7
7030	Adult	Male	122.3	154.7	165.4
7031	Sub-adult	Female	1.4	5.2	0.4
7032	Adult	Male	5.6	3.1	6.4

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
7014	Adult	Male	58.3	83.6	49.8
7015	Adult	Female	75.1	36.8	79.7
SM522	Adult	Female	41.4	26.2	47.8
SM557	Adult	Male	6.6	13.4	5.4
SM607	Adult	Male	6.1	3.1	6.3
SM626	Adult	Male	30.3	10.0	35.1
SM659	Adult	Male	8.6	8.7	8.6
SMA109	Adult	Female	9.9	7.8	10.2
SMA905	Adult	Female	13.5	13.9	13.4
SMA906	Sub-adult	Female	26.7	26.2	28.6
SMB012	Adult	Male	7.4	22.9	5.0
SMB172	Adult	Male	25.8	26.6	28.7
SMB469*	Adult	Female	133.2	114.4	124.8
SMB885*	Adult	Male	77.7	103.2	96.5

Abbreviations: ID – identification number; MARV – Marburg virus; GP – glycoprotein; NP – nucleoprotein; ELISA – enzyme-linked immunosorbent assay; PP – percent positivity

[a] Cut-off for MARV GP ELISA – 17.1 PP

[b] Cut-off for MARV NP ELISA – 25.7 PP

[c] Cut-off for commercial MARV GP ELISA – 16.8 PP

*Also used for dose/response curves

PANEL 7

Description: Wild-caught bats brought into captivity for NICD breeding colony. Previously tested using an I-ELISA based on commercially available recombinant MARV GP. Used for assay evaluation (robustness, repeatability, dose/response curves, intermediate precision).

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
F6F	Adult	Female	19.2	26.1	20.1

Bat ID	Age	Sex	MARV GP ELISA result (PP)[a]	MARV NP ELISA result (PP)[b]	Commercial MARV GP ELISA result (PP)[c]
14/04/20	Adult	Female	11.5	11.4	10.9
SMB492	Adult	Male	168.5	159.9	177.2
SMB489	Adult	Female	34.8	41.5	35.4
SMB461	Adult	Male	17.4	27.3	20.5
SMB565	Adult	Female	75.2	162.1	71.3
SMB416	Adult	Male	12.4	12.5	11.9
14/04/31	Adult	Male	9.2	7.9	7.6
6211	Adult	Female	14.7	12.9	11.8
14/04/18	Adult	Female	10.4	16.6	9.8
SMB552	Adult	Female	31.9	35.9	36.1

Abbreviations: ID – identification number; MARV – Marburg virus; GP – glycoprotein; NP – nucleoprotein; ELISA – enzyme-linked immunosorbent assay; PP – percent positivity

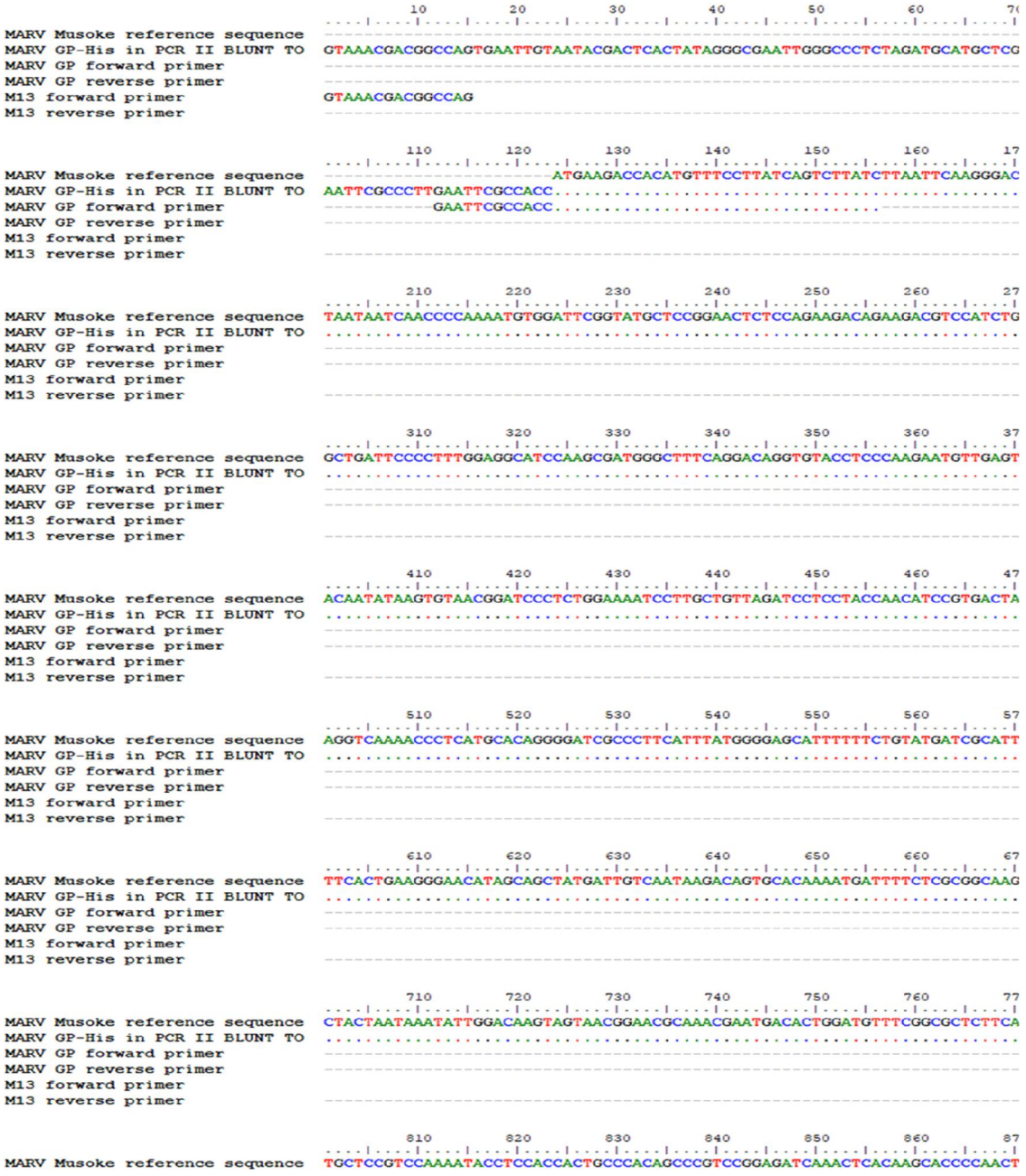
[a] Cut-off for MARV GP ELISA – 17.1 PP

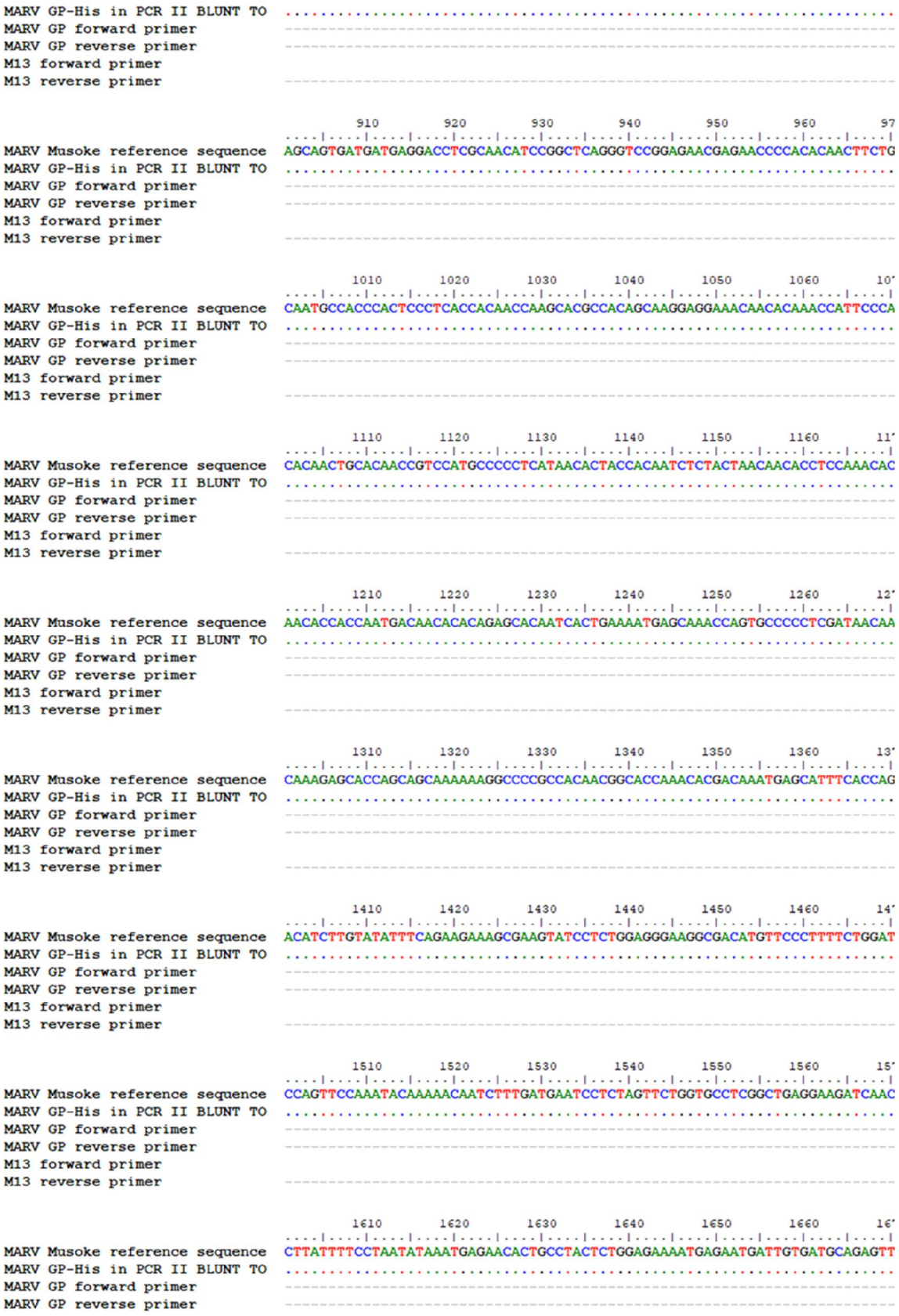
[b] Cut-off for MARV NP ELISA – 25.7 PP

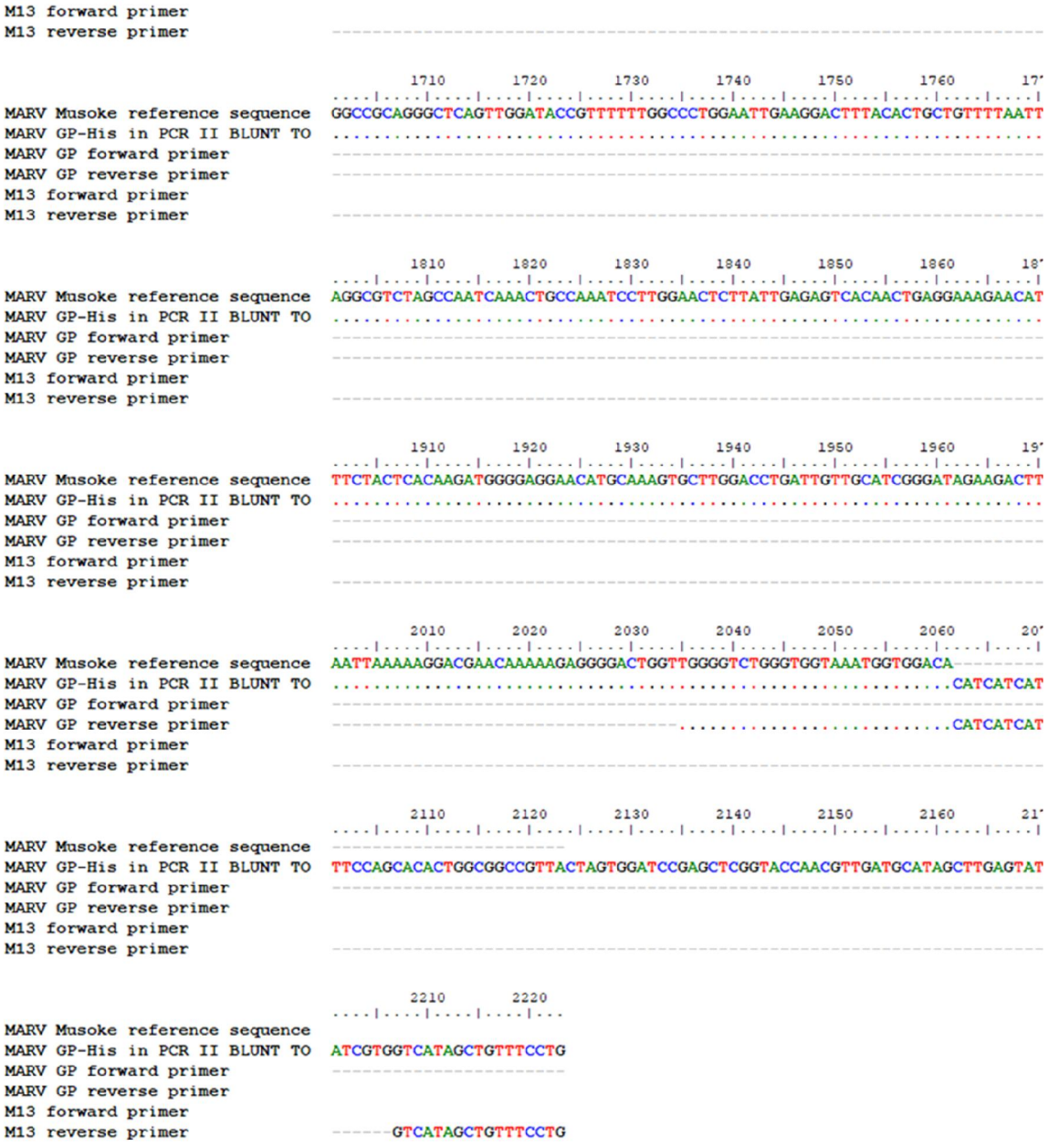
[c] Cut-off for commercial MARV GP ELISA – 16.8 PP

APPENDIX C

Multiple alignment produced from sequencing information of the MARV Musoke GP-His in the PCR-II Blunt-TOPO vector and MARV Musoke reference strain GP (available in the public domain, accession number NC_001608.3) using the ClustalW subroutine of the BioEdit Sequence Alignment Editor, version 7.2.5

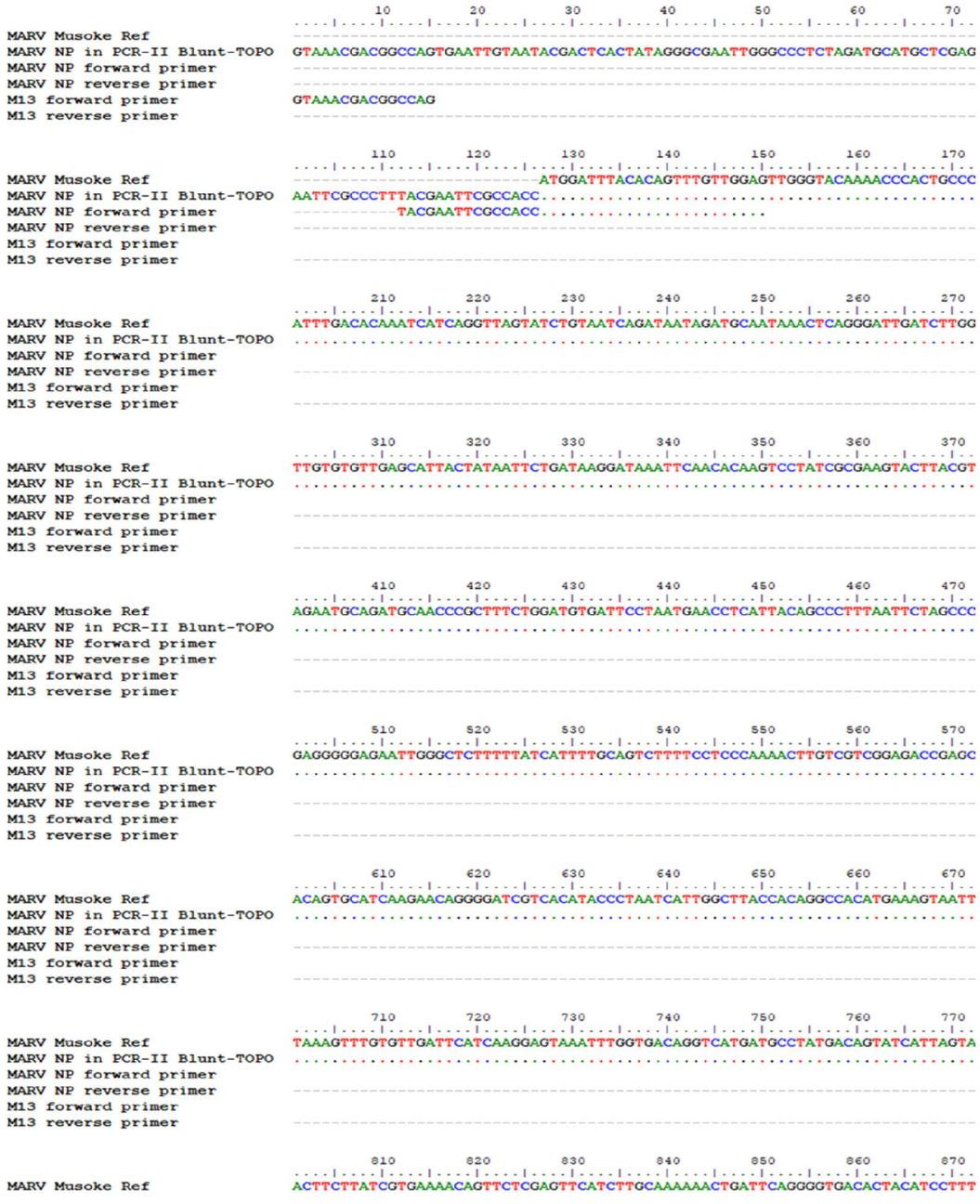


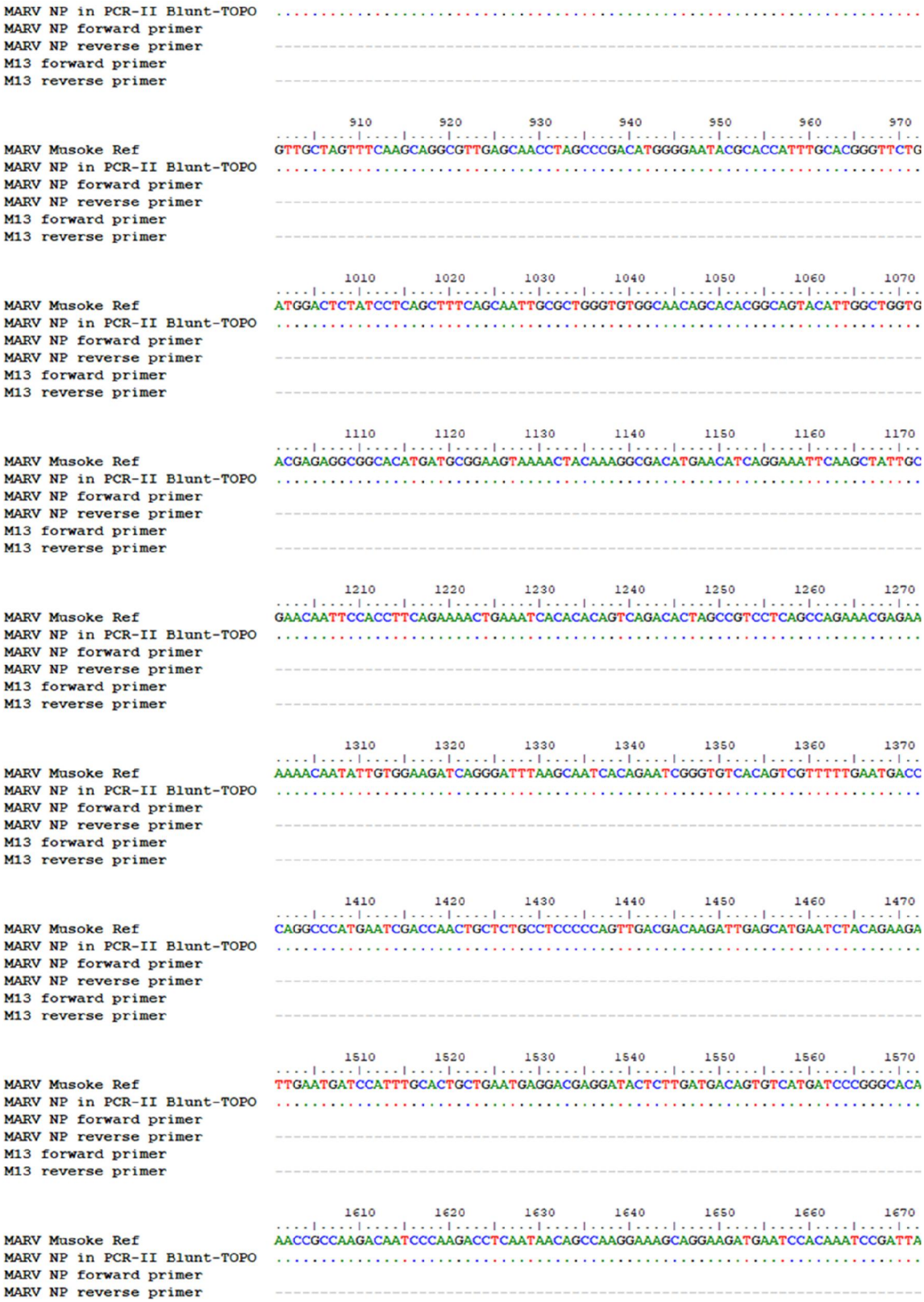

MARV GP-His in PCR II BLUNT TO
MARV GP forward primer
MARV GP reverse primer
M13 forward primer
M13 reverse primer
910 920 930 940 950 960 97
MARV Musoke reference sequence AGCAGTGATGATGAGGACCTCGCAACATCCGGCTCAGGGTCCGGAGAACGAGAAACCCCACACAACTTCTG
1010 1020 1030 1040 1050 1060 10
MARV Musoke reference sequence CAATGCCACCCACTCCCTCACCACAACCAAGCACGCCACAGCAAGGAGGAAACAACACAAACCATTCCCA
1110 1120 1130 1140 1150 1160 11
MARV Musoke reference sequence CACAACTGCACAACCGTCCATGCCCCCTCATAACACTACCACAATCTCTACTAACAACACCTCCAAACAC
1210 1220 1230 1240 1250 1260 12
MARV Musoke reference sequence AACACCACCAATGACAACACACAGAGCACAATCACTGAAAATGAGCAAACCAGTGCCCCCTCGATAACAA
1310 1320 1330 1340 1350 1360 13
MARV Musoke reference sequence CAAAGAGCACCAGCAGCAAAAAAGGCCCCGCCACAACGGCACCAAACACGACAAATGAGCATTTCACCAG
1410 1420 1430 1440 1450 1460 14
MARV Musoke reference sequence ACATCTTGTATATTTCAGAAGAAAGCGAAGTATCCTCTGGAGGGAAGGCGACATGTTCCCTTTTCTGGAT
1510 1520 1530 1540 1550 1560 15
MARV Musoke reference sequence CCAGTTCCAAATACAAAAACAATCTTTGATGAATCCTCTAGTTCTGGTGCCTCGGCTGAGGAAGATCAAC
1610 1620 1630 1640 1650 1660 16
MARV Musoke reference sequence CTTATTTTCCTAATATAAATGAGAACACTGCCTACTCTGGAGAAAATGAGAATGATTGTGATGCAGAGTT

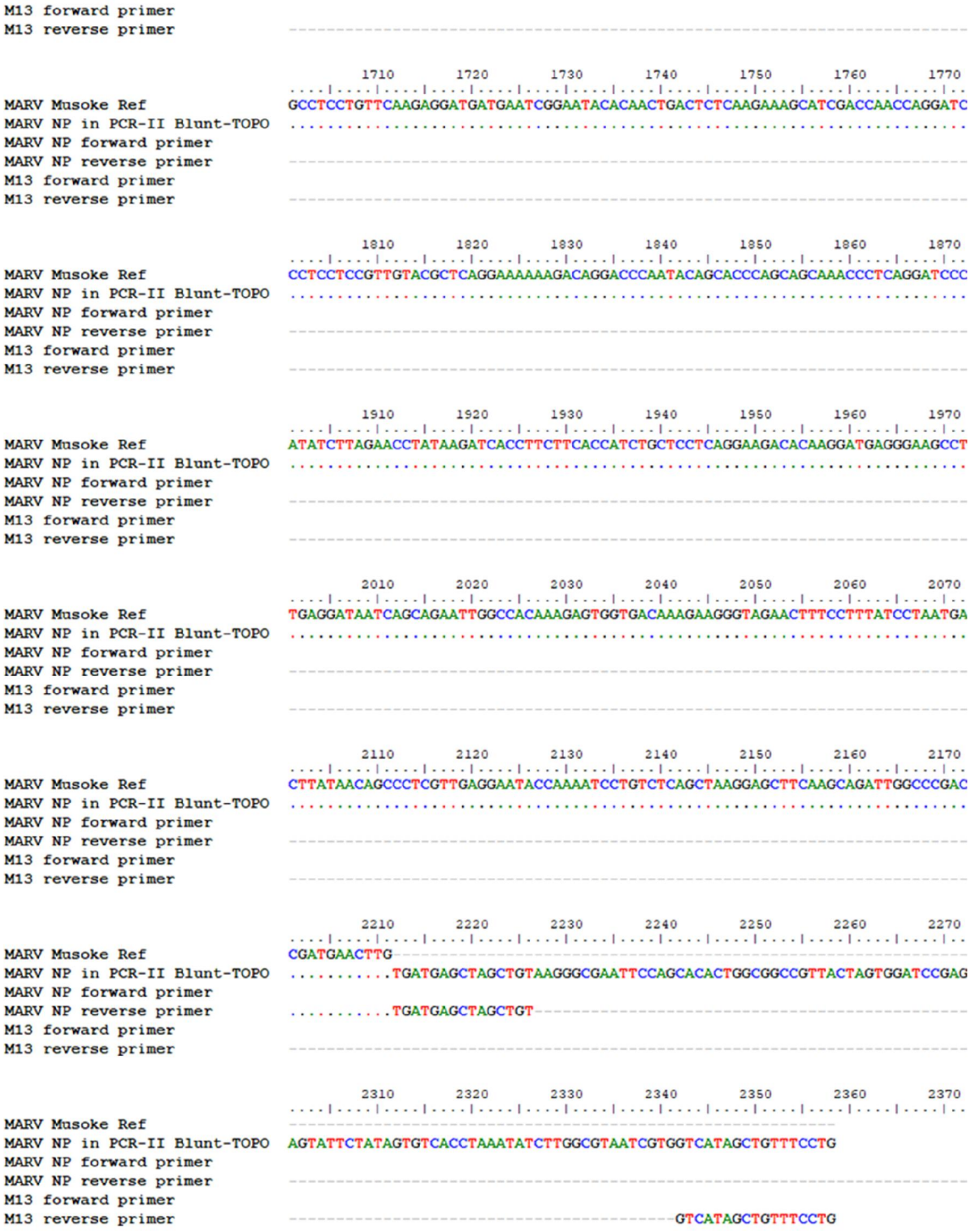

M13 forward primer
M13 reverse primer
1710 1720 1730 1740 1750 1760 17
MARV Musoke reference sequence GGCCGCAGGGCTCAGTTGGATACCGTTTTTTGGCCCTGGAATTGAAGGACTTTACACTGCTGTTTTAATT
MARV GP-His in PCR II BLUNT TO
MARV GP forward primer
MARV GP reverse primer
M13 forward primer
M13 reverse primer
1810 1820 1830 1840 1850 1860 18
MARV Musoke reference sequence AGGCGTCTAGCCAATCAAACTGCCAAATCCTTGGAACTCTTATTGAGAGTCACAACTGAGGAAAGAACAT
MARV GP-His in PCR II BLUNT TO
MARV GP forward primer
MARV GP reverse primer
M13 forward primer
M13 reverse primer
1910 1920 1930 1940 1950 1960 19
MARV Musoke reference sequence TTCTACTCACAAGATGGGGAGGAACATGCAAAGTGCTTGGACCTGATTGTTGCATCGGGATAGAAGACTT
MARV GP-His in PCR II BLUNT TO
MARV GP forward primer
MARV GP reverse primer
M13 forward primer
M13 reverse primer
2010 2020 2030 2040 2050 2060 20
MARV Musoke reference sequence AATTAAAAAGGACGAACAAAAAGAGGGGACTGGTTGGGGTCTGGGTGGTAAATGGTGGACA
MARV GP-His in PCR II BLUNT TO CATCATCAT
MARV GP forward primer
MARV GP reverse primer CATCATCAT
M13 forward primer
M13 reverse primer
2110 2120 2130 2140 2150 2160 21
MARV Musoke reference sequence
MARV GP-His in PCR II BLUNT TO TTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAGCTCGGTACCAACGTTGATGCATAGCTTGAGTAT
MARV GP forward primer
MARV GP reverse primer
M13 forward primer
M13 reverse primer
2210 2220
MARV Musoke reference sequence
MARV GP-His in PCR II BLUNT TO ATCGTGGTCATAGCTGTTTCCTG
MARV GP forward primer
MARV GP reverse primer
M13 forward primer
M13 reverse primer GTCATAGCTGTTTCCTG

APPENDIX D

Multiple alignment produced from sequencing information of the MARV Musoke NP in the PCR-II Blunt-TOPO vector and MARV Musoke reference strain NP (available in the public domain, accession number NC_001608.3) using the ClustalW subroutine of the BioEdit Sequence Alignment Editor, version 7.2.5




MARV NP in PCR-II Blunt-TOPO
MARV NP forward primer
MARV NP reverse primer
M13 forward primer
M13 reverse primer
910 920 930 940 950 960 970
MARV Musoke Ref
GTTGCTAGTTTCAAGCAGGCGTTGAGCAACCTAGCCCGACATGGGGAATACGCACCATTTGCACGGGTTCTG
1010 1020 1030 1040 1050 1060 1070
ATGGACTCTATCCTCAGCTTTCAGCAATTGCGCTGGGTGTGGCAACAGCACACGGCAGTACATTGGCTGGTG
1110 1120 1130 1140 1150 1160 1170
ACGAGAGGCGGCACATGATGCGGAAGTAAAACTACAAAGGCGACATGAACATCAGGAAATTCAAGCTATTGC
1210 1220 1230 1240 1250 1260 1270
GAACAATTCCACCTTCAGAAAACTGAAATCACACACAGTCAGACACTAGCCGTCCTCAGCCAGAAACGAGAA
1310 1320 1330 1340 1350 1360 1370
AAAACAATATTGTGGAAGATCAGGGATTTAAGCAATCACAGAATCGGGTGTCACAGTCGTTTTTGAATGACC
1410 1420 1430 1440 1450 1460 1470
CAGGCCCATGAATCGACCAACTGCTCTGCCTCCCCCAGTTGACGACAAGATTGAGCATGAATCTACAGAAGA
1510 1520 1530 1540 1550 1560 1570
TTGAATGATCCATTTGCACTGCTGAATGAGGACGAGGATACTCTTGATGACAGTGTCATGATCCCGGGCACA
1610 1620 1630 1640 1650 1660 1670
AACCGCCAAGACAATCCCAAGACCTCAATAACAGCCAAGGAAAGCAGGAAGATGAATCCACAAATCCGATTA


M13 forward primer
M13 reverse primer
1710 1720 1730 1740 1750 1760 1770
MARV Musoke Ref GCCTCCTGTTCAAGAGGATGATGAATCGGAATACACAACTGACTCTCAAGAAAGCATCGACCAACCAGGATC
MARV NP in PCR-II Blunt-TOPO
MARV NP forward primer
MARV NP reverse primer
M13 forward primer
M13 reverse primer
1810 1820 1830 1840 1850 1860 1870
MARV Musoke Ref CCTCCTCCGTTGTACGCTCAGGAAAAAAGACAGGACCCAATACAGCACCCAGCAGCAAACCCTCAGGATCCC
MARV NP in PCR-II Blunt-TOPO
MARV NP forward primer
MARV NP reverse primer
M13 forward primer
M13 reverse primer
1910 1920 1930 1940 1950 1960 1970
MARV Musoke Ref ATATCTTAGAACCTATAAGATCACCTTCTTCACCATCTGCTCCTCAGGAAGACACAAGGATGAGGGAAGCCT
MARV NP in PCR-II Blunt-TOPO
MARV NP forward primer
MARV NP reverse primer
M13 forward primer
M13 reverse primer
2010 2020 2030 2040 2050 2060 2070
MARV Musoke Ref TGAGGATAATCAGCAGAATTGGCCACAAAGAGTGGTGACAAAGAAGGGTAGAACTTTCCTTTATCCTAATGA
MARV NP in PCR-II Blunt-TOPO
MARV NP forward primer
MARV NP reverse primer
M13 forward primer
M13 reverse primer
2110 2120 2130 2140 2150 2160 2170
MARV Musoke Ref CTTATAACAGCCCTCGTTGAGGAATACCAAAATCCTGTCTCAGCTAAGGAGCTTCAAGCAGATTGGCCCGAC
MARV NP in PCR-II Blunt-TOPO
MARV NP forward primer
MARV NP reverse primer
M13 forward primer
M13 reverse primer
2210 2220 2230 2240 2250 2260 2270
MARV Musoke Ref CGATGAACTTG
MARV NP in PCR-II Blunt-TOPO ..........TGATGAGCTAGCTGTAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCCGAG
MARV NP forward primer
MARV NP reverse primer ..........TGATGAGCTAGCTGT
M13 forward primer
M13 reverse primer
2310 2320 2330 2340 2350 2360 2370
MARV Musoke Ref
MARV NP in PCR-II Blunt-TOPO AGTATTCTATAGTGTCACCTAAATATCTTGGCGTAATCGTGGTCATAGCTGTTTCCTG
MARV NP forward primer
MARV NP reverse primer
M13 forward primer
M13 reverse primer GTCATAGCTGTTTCCTG

APPENDIX E

viruses

MDPI

Article

Antibody Responses to Marburg Virus in Egyptian Rousette Bats and Their Role in Protection against Infection

Nadia Storm [1,2], Petrus Jansen van Vuren [1,3], Wanda Markotter [3] and Janusz T. Paweska [1,3,4,*]

1 Centre for Emerging Zoonotic and Parasitic Diseases, National Institute for Communicable Diseases of the National Health Laboratory Service, Sandringham, Johannesburg 2131, South Africa; nadias@nicd.ac.za (N.S.); petrusv@nicd.ac.za (P.J.v.V.)
2 Department of Microbiology and Plant Pathology, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria 0002, South Africa
3 Centre for Viral Zoonoses, Department of Medical Virology, School of Medicine, Faculty of Health Sciences, University of Pretoria, Pretoria 0001, South Africa; wanda.markotter@up.ac.za
4 Division Virology and Communicable Diseases Surveillance, School of Pathology, University of the Witwatersrand, Johannesburg 2193, South Africa
* Correspondence: januszp@nicd.ac.za; Tel.: +27-11-386-6336

Received: 9 January 2018; Accepted: 2 February 2018; Published: 10 February 2018

Abstract: Egyptian rousette bats (ERBs) are reservoir hosts for the Marburg virus (MARV). The immune dynamics and responses to MARV infection in ERBs are poorly understood, and limited information exists on the role of antibodies in protection of ERBs against MARV infection. Here, we determine the duration of maternal immunity to MARV in juvenile ERBs, and evaluate the duration of the antibody response to MARV in bats naturally or experimentally infected with the virus. We further explore whether antibodies in previously naturally exposed bats is fully protective against experimental reinfection with MARV. Maternal immunity was lost in juvenile ERBs by 5 months of age. Antibodies to MARV remained detectable in 67% of experimentally infected bats approximately 4 months post inoculation (p.i.), while antibodies to MARV remained present in 84% of naturally exposed bats at least 11 months after capture. Reinfection of seropositive ERBs with MARV produced an anamnestic response from day 5 p.i. Although PCR-defined viremia was present in 73.3% of reinfected ERBs, replicating virus was recovered from the serum of only one bat on day 3 p.i. The negative PCR results in the salivary glands, intestines, bladders and reproductive tracts of reinfected bats, and the apparent absence of MARV in the majority of swabs collected from these bats suggest that reinfection may only play a minor role in the transmission and maintenance of MARV amongst ERBs in nature.

Keywords: Marburg virus; Egyptian rousette bat; antibody response; maternal immunity; immune duration; reinfection; viral shedding; South Africa

1. Introduction

Marburg virus (MARV) is a member of the family *Filoviridae* and is the causative agent of severe and often fatal hemorrhagic fever in humans and non-human primates [1]. At least 13 outbreaks of MARV disease have been recorded to date, of which several have been associated with entry into caves or mines, or contact with bats [2–9]. The Egyptian rousette bat (ERB), *Rousettus aegyptiacus*, has been recognized as a reservoir host for MARV based on repeated isolation of the virus from naturally infected bats [10–12] as well as the absence of clinical disease following experimental inoculation [13–16]. Much progress has been made in identifying and studying the reservoir host for

MARV in recent years [10–18], but knowledge of the biology of MARV within ERBs remains sparse. Spillover of MARV into human and animal populations often coincides with periods of increased viral shedding from ERBs [11], but the mechanisms driving the transmission and maintenance of MARV remain to be described. Currently, three hypotheses for MARV transmission dynamics in ERB populations exist: (1) bats may obtain lifelong immunity following recovery from a primary infection, and new outbreaks of the virus only occur when the pool of susceptible bats is replenished by weaned juveniles that have lost maternal immunity; (2) immunity to MARV in bats may be transient, with the virus being able to persist through fluctuating herd immunity; or (3) bats may be persistently infected with MARV, shedding virus periodically due to physiological or environmental stress factors [19]. Mathematical models and longitudinal ecological studies of filoviruses in ERBs have suggested that seasonal increases in viral shedding may occur in response to drivers such as waning immunity, births and migration of bats between colonies [11,20,21]. At present, the only evidence available points to the loss of maternal immunity in juvenile bats as being a major driver of MARV maintenance in nature [11], but information on exactly when juveniles lose maternal immunity and become susceptible to infection with the virus is limited.

Bat immunity is a poorly studied subject, and little is known about the role of antibody responses in the protection of ERBs against MARV infection. It also remains unclear whether primary infection with MARV results in lifelong, long term or transient protective immunity. A study by Schuh et al. recently showed that antibodies against MARV in experimentally infected ERBs declined to undetectable levels by the third month post experimental infection [16], possibly resulting in the susceptibility of these bats to MARV reinfection. Other studies have shown that antibodies may not be a major driver of viral clearance in bats [22,23], and that antibodies may even enhance MARV infection in vitro [24].

Determining the duration of actively and passively acquired immunity in ERBs, and whether actively acquired anti-MARV antibodies in ERBs are protective against reinfection, may assist in understanding how herd immunity influences MARV maintenance and population transmission dynamics. This knowledge may, in turn, assist in predicting and preventing spillover events into human and other animal populations. In this study, we show that maternal antibodies to MARV are lost in juvenile ERBs between 4 and 5 months after birth, becoming susceptible to primary infection with the virus. We also show that actively acquired antibodies to MARV in ERBs experimentally or naturally infected with the virus remain detectable in the majority of bats at 110 days post infection (67%) and 11 months after capture (84%), respectively, contrasting with the results found by Schuh et al. [16]. In a previous study, we demonstrated short term protective immunity against MARV reinfection and replication in ERBs first inoculated 48 days prior [15]. Here, we determine the role of the antibody response in the protection of previously naturally exposed ERBs against MARV reinfection. We show that antibodies do not completely protect previously MARV-exposed bats against reinfection, but effectively curbs systemic spread of the virus. The resulting lack of viral shedding implies that reinfection of previously exposed bats is not a major contributor to the transmission and maintenance dynamics of MARV in ERBs in nature.

2. Materials and Methods

2.1. Regulatory Requirements and Ethics Clearance

Egyptian rousette bats were sourced, housed and handled as previously described [13,15]. A permit for the capturing of ERBs in the Limpopo Province of South Africa (005-00002, 3-5-2012) was obtained from the Limpopo Department of Economic Development, Environment and Tourism as well as the Gauteng Department of Agriculture and Rural Development (003767, 25-5-2012). Approval for establishing an ERB breeding colony and performing experimental infections of ERBs with MARV was obtained from the Department of Agriculture, Forestry and Fisheries of South Africa (12/11/1/1/13, 4-2-2013). Ethics approval for the colonization and experimental infection of ERBs with MARV

was acquired from the National Health Laboratory Service Animal Ethics Committee (AEC 136/12, 12-12-2012; AEC 139/13, 30-8-2013), as well as the University of Pretoria Animal Ethics Committee (EC056-14, 30-6-2014; H018-16, 28-11-2016).

2.2. Experiment 1: Duration of Maternal Immunity to Marburg Virus in Juvenile Egyptian Rousette Bats

Twenty-six juvenile ERBs were captured using harp traps and brought into captivity as previously described [13]. Upon capture, juvenile status was confirmed by observing a lack of epiphyseal-diaphyseal fusion of the long phalanges, as well as juvenile size and pelage [25]. The bats were bled on four separate occasions using previously described methods [13,15]. At the first sampling, the bats were weighed and their forearms measured using a vernier caliper (dialMax, Wiha Tools Ltd., Worcestershire, UK). The age of each bat was then estimated according to the forearm length growth curve published by Mutere [26]. In addition to wild-caught juvenile bats, 20 bats born in captivity to MARV-seropositive dams were bled and tested for the presence of maternal antibodies on five separate occasions, with sampling commencing at 3 months of age. Sera were separated from the blood samples collected from the ERBs by centrifugation at 3000× *g* for 10 min and were tested for anti-MARV immunoglobulin G (IgG) antibodies using an indirect enzyme-linked immunosorbent assay (I-ELISA). The I-ELISA was based on the recombinant MARV (Musoke) glycoprotein (GP) antigen produced in a mammalian expression system and was performed under biosafety level four conditions as previously described [15]. The negative control serum, conjugate controls and test sera were assayed in duplicate at a dilution of 1:100, and positive control serum was assayed in quadruplicate. Positive control serum was derived from a pool of serum from ERBs infected with MARV during a previous experiment [13], and negative control serum was derived from a pool of serum obtained from six MARV-naive ERBs born in captivity. Optical density (OD) values were measured at 405 nm using a microplate reader. The means of the OD values of the test sera replicates were calculated and converted to a percentage positivity (PP) relative to the positive control serum using the following equation: (mean net OD of test sera replicates/mean net OD of positive control) × 100 [27].

2.3. Experiment 2: Duration of the Antibody Response to Marburg Virus in Experimentally Infected Egyptian Rousette Bats

Six MARV-naive ERBs were inoculated subcutaneously with 100 µL of tissue culture supernatant containing $10^{5.3}$/mL tissue culture infectious dose ($TCID_{50}$) of MARV (isolate SPU 148/99/I Watsa, second passage in Vero cells). The bats were clinically monitored and bled over a period of 110 days as previously described [13,15]. Sera were tested for anti-MARV IgG antibodies using I-ELISA as previously described [15].

2.4. Experiment 3: Duration of the Antibody Response to Marburg Virus in Naturally Infected Egyptian Rousette Bats

Thirty-eight bats that had previously been exposed to MARV in nature as evidenced by the presence of anti-MARV IgG antibodies in sera at the time of capture (PP value range: 22.6–176.1; cut-off value for I-ELISA: 16.8 PP) were brought into captivity as previously described [13]. The bats were monitored for the presence of anti-MARV IgG antibodies in their sera over a period of 11 months using I-ELISA as previously described [15].

2.5. Experiment 4: Re-Infection of Seropositive Egyptian Rousette Bats with Marburg Virus

2.5.1. Inoculation of Seropositive Egyptian Rousette Bats with Marburg Virus

Seventeen wild-caught bats with MARV-specific IgG PP values ranging from 26.5 to 146.3 were selected for this experiment. Bat sera were tested for the presence of antibodies to MARV both at capture and one week prior to commencing the experiment. While the infection histories of the bats were unknown, the bats had most likely been infected with the MARV variant circulating in a cave

located in the Matlapitsi Valley, Limpopo Province, South Africa [28], where the bats were captured. Fifteen bats (13 adult females and two adult males) were inoculated subcutaneously with 100 µL of tissue culture supernatant containing $10^{5.3}$/mL $TCID_{50}$ of MARV/Hsap/COD/99/Watsa-SPU148-99-I, (second passage in Vero cells). Two control bats (adult females) were inoculated subcutaneously with 100 µL of Eagle's Minimum Essential Medium (EMEM; Lonza, Basel, Switzerland). Bats were clinically monitored daily, and swabbed, bled and serially euthanized on days 0, 3, 5, 7, 9 and 12 post inoculation (p.i.) as previously described [13,15]. Results from this experiment were compared to the results obtained from naive experimentally infected bats in a previous study by our group [15].

2.5.2. Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction and Virus Isolation

Ribonucleic acid (RNA) was extracted from serum, swabs and the supernatant of 10% tissue homogenates in EMEM (Lonza, Basel, Switzerland) using the QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), calculation of RNA copy numbers and conversion of copy numbers to $TCID_{50}$ equivalents in samples tested were performed as previously described [13]. Virus isolation was attempted on all specimens with qRT-PCR positive results and was performed as previously described [13].

2.5.3. Virus Neutralization Index

In order to evaluate the ability of anti-MARV IgG in sera from bats naturally exposed to a local strain of MARV (see Section 2.5.1) to neutralize a genetically distinct MARV strain, a virus neutralization index test was performed. Briefly, ten-fold dilutions of MARV/Hsap/COD/99/Watsa-SPU148-99-I (second passage in Vero cells), and MARV/Hsap/ZAF/75/Ozolin (fourth passage in Vero cells), were prepared in 96 well cell culture plates (NUNC) in sextuplicate. Triplicates of each ten-fold dilution was mixed with a 1:20 dilution of pooled MARV-positive or -negative bat serum in Minimum Essential Medium (MEM) Rega-3 (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and the plate was incubated at 37 °C for 1 hour. A confluent flask of human adrenal carcinoma cells (SW-13) was trypsinized into 40 mL MEM Rega-3 containing 8% fetal calf serum (HyClone, Separations, Randburg, Gauteng, South Africa). One hundred microliters of the cell suspension was added to each well of the plate, and the plate was incubated at 37 °C in a 5% CO_2 atmosphere for 7 days. The plate was fixed in 80% acetone (Sigma-Aldrich, St. Louis, MI, USA) and the foci stained with rabbit anti-MARV serum followed by a fluorescein isothiocyanate-labeled secondary antibody (Sigma-Aldrich, St. Louis, MI, USA). Fluorescent foci were observed using a fluorescence microscope (EVOS, Tampa, FL, USA) and the titers of the neutralized and non-neutralized viruses were determined using the method of Spearman and Karber [29].

2.6. Statistical Analysis

All statistical tests were performed in Microsoft Excel. Correlation between ELISA PP value and levels of equivalent viremia in reinfected bats was determined using Spearman's Rank-Order Correlation. The Student's *t*-test was performed to determine whether anti-MARV IgG levels differed significantly between naive [15] and seropositive MARV infected bats (two-tailed p-value < 0.05). The statistical significance of differences in viral load in the tissues of naive and seropositive infected bats was determined by performing the Kruskal-Wallis rank test.

3. Results

3.1. Experiment 1: Duration of Maternal Immunity to Marburg Virus in Egyptian Rousette Bats

All wild-caught juveniles tested in this study were born to dams previously naturally infected with MARV as evidenced by the presence of MARV-specific maternal IgG antibodies in the juveniles (26/26) at the time of the first sampling (Figure 1). The average age of the juvenile ERBs at the first

bleed was 8 weeks (range: 6–10 weeks). By the second bleed (approximately 3 months after birth), the percentage of juveniles with detectable maternal IgG antibodies to MARV had declined to 50% (13/26). At approximately 5 months after birth, maternal IgG antibodies to MARV could only be detected in a single bat, and by 7 months after birth, none of the bats had detectable levels of maternal IgG antibodies to MARV. Similarly, no maternal antibodies could be detected in juvenile bats born from captive MARV-seropositive dams at 5 and 8 months of age, even though maternal antibody titers to MARV in these bats at 3 months of age were much higher than those of wild-caught juvenile bats at a comparable age (Figure 1).

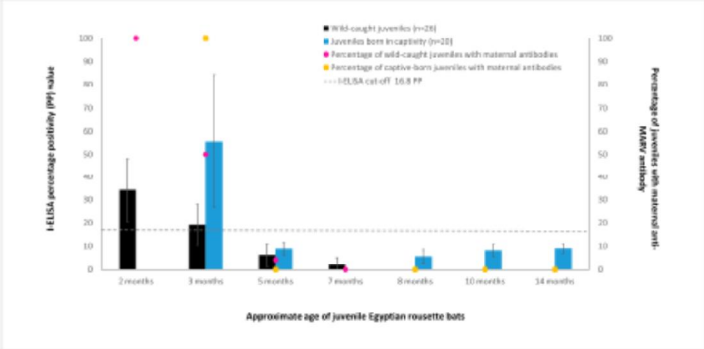


Figure 1. Mean maternal anti-Marburg virus (MARV) IgG antibody levels in juvenile bats born from naturally exposed mothers, with error bars representing the standard deviation of the measurements. ELISA results are shown as the percentage positivity (PP) in relation to the positive control serum. The dashed grey line represents the cut-off value of the assay at 16.8 PP (left-hand *y*-axis). The percentage of juveniles with maternal anti-MARV antibodies is displayed on the right-hand *y*-axis.

3.2. Experiment 2: Duration of the Antibody Response to Marburg Virus in Experimentally Infected Egyptian Rousette Bats

Immunoglobulin G antibodies to MARV peaked in all experimentally infected bats at day 14 p.i. (Figure 2), and then started to decline towards day 110 p.i. There was considerable variation in the immune responses of each bat, with 3 of the 6 bats producing IgG antibodies with a maximum average ELISA PP value of only 41.2. In 2 of these bats, IgG antibodies declined to undetectable levels by day 110 p.i., but IgG antibodies could still be detected in 4 of the 6 bats (67%) on this day.

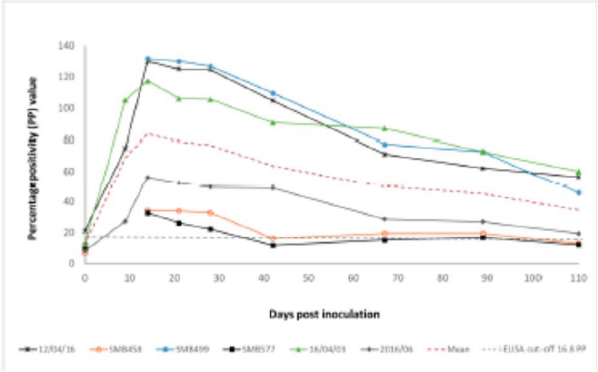


Figure 2. Duration of the IgG immune response to Marburg virus in individual experimentally infected Egyptian rousette bats (n = 6), with the dashed red line representing the mean duration of the IgG immune response. ELISA results are shown as the percentage positivity (PP) in relation to the positive control serum. The dashed grey line represents the cut-off value of the assay at 16.8 PP.

3.3. Experiment 3: Duration of the Antibody Response to Marburg Virus in Naturally Infected Egyptian Rousette Bats

Immunoglobulin G antibodies to MARV in previously naturally exposed bats gradually declined over a period of 11 months (Figure 3). Marburg virus-specific antibodies became undetectable in only 6 of the 38 bats (15.8%) between month 9 and 11 after capture.

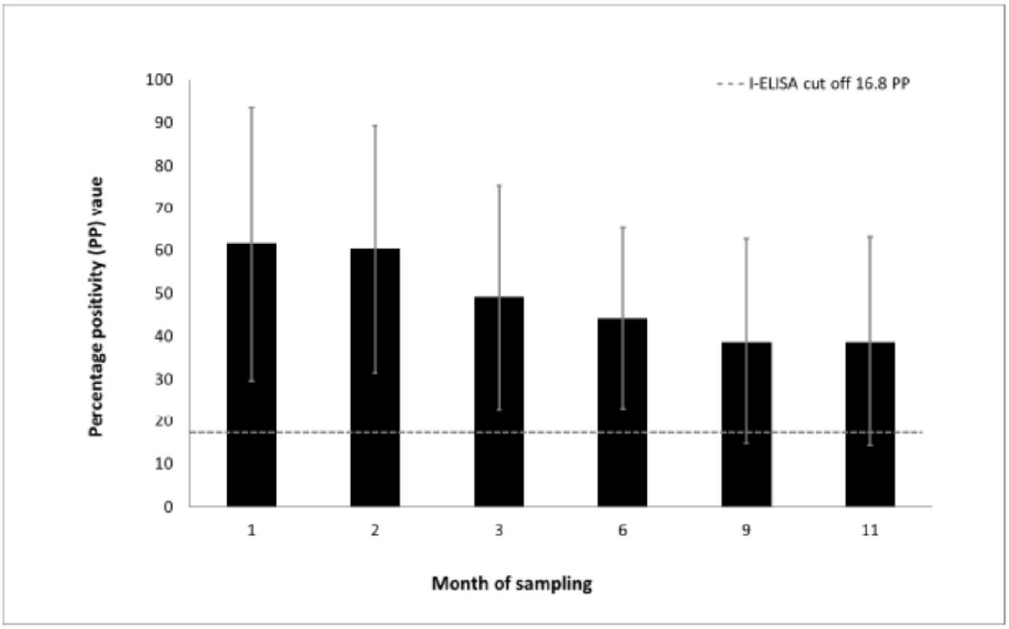


Figure 3. Mean duration of the IgG immune response to Marburg virus in previously naturally exposed Egyptian rousette bats (n = 38), with error bars representing the standard deviation of the measurements. ELISA results are shown as the percentage positivity (PP) in relation to the positive control serum. The dashed grey line represents the cut-off value of the assay at 16.8 PP.

3.4. Experiment 4: Re-Infection of Seropositive Egyptian Rousette Bats with Marburg Virus

3.4.1. Serology

There were no apparent signs of morbidity or mortality in any of the MARV-inoculated or control bats for the duration of the experiment. There was a statistically significant difference between the ELISA PP values of naive [15] and seropositive bats both before (two-tailed p-value: 0.00001), and after inoculation with MARV (two-tailed p-value: 0.0002). A substantial boosting effect of MARV infection on the anti-MARV GP IgG levels was noted in the MARV-inoculated bats from day 5 p.i. (Figure 4). The anti-MARV IgG levels in control bats remained unchanged for the duration of this study.

3.4.2. Detection of MARV RNA by qRT-PCR and Virus Isolation

Based on qRT-PCR, 11 of the 15 seropositive bats (73.3%) were viremic on the third day p.i. (Table 1), similar to findings in naive bats infected with MARV [15]. However, the challenge virus was isolated from only one qRT-PCR positive serum (bat 4, $10^{1.08}$ $TCID_{50}$/mL, ELISA PP value 47.5) on day 3 p.i. Replication of the virus in serum was also demonstrated in one additional bat (bat 2, ELISA PP value 26.5), with the level of equivalent viremia increasing from $10^{0.7}$ $TCID_{50}$/mL on day 3 p.i. to $10^{1.38}$ $TCID_{50}$/mL on day 5 p.i. Unlike in naive infected bats [15], MARV could not be detected in the blood of seropositive bats from day 7 p.i. Marburg virus concentrations in the serum of seropositive bats on day 3 p.i. ranged from $10^{-0.09}$ $TCID_{50}$/mL to $10^{2.3}$ $TCID_{50}$/mL. In comparison, MARV concentrations in the serum of naive infected bats [15] on day 3 p.i. ranged from $10^{-0.3}$ $TCID_{50}$/mL to $10^{2.1}$ $TCID_{50}$/mL,

with no statistically significant differences between the levels of equivalent viremia in naive [15] and seropositive bats on this day (two-tailed *p*-value: 0.74).

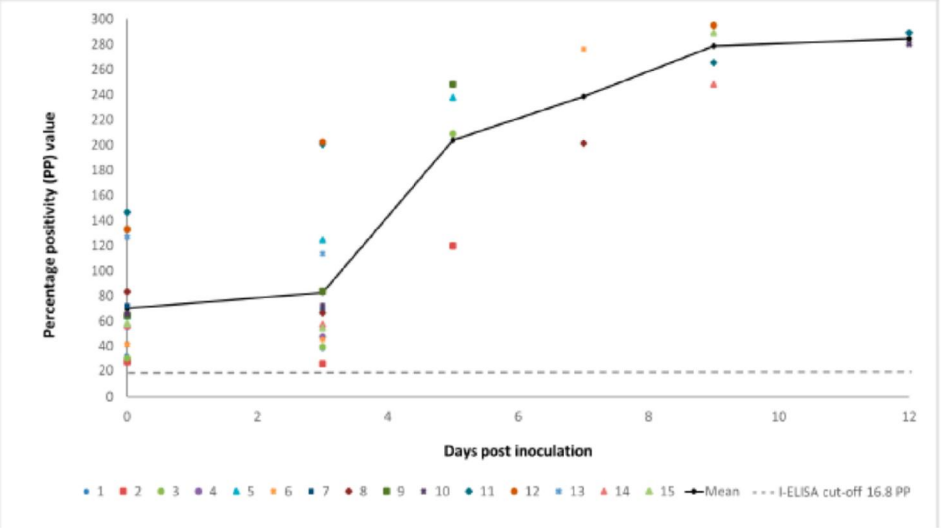


Figure 4. Immunoglobulin G antibody responses in 15 Egyptian rousette bats with pre-existing natural immunity following experimental infection with Marburg virus. ELISA results are shown as the percentage positivity (PP) in relation to the positive control serum. The dashed grey line represents the cut-off value of the assay at 16.8 PP.

Table 1. Quantitative reverse-transcription polymerase chain reaction and virus isolation results in specimens from seropositive Egyptian rousette bats experimentally inoculated with Marburg virus.

	Days after Inoculation [a]				
	3 (*n* = 15)	**5 (*n* = 3)**	**7 (*n* = 3)**	**9 (*n* = 6)**	**12 (*n* = 2)**
Bat IDs	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15	2, 5, 9	3, 6, 8	10, 11, 12, 13, 14, 15	11, 15
Specimen					
Serum	11/15; VI: 1/11	1/3; VI: 0/1	0/3	0/6	0/2
Rectal swab	0/15	0/3	0/3	0/6	0/2
Nasal swab	0/15	1/3; VI: 0/1	0/3	0/6	0/2
Oral swab	0/15	0/3	0/3	0/6	0/2
Vaginal swab	NS	NS	NS	0/4	0/1
Penile swab	NS	NS	NS	0/2	0/1
Liver	2/3; VI: 0/2	3/3; VI: 0/3	0/3	1/4; VI: 0/1	0/2
Spleen	3/3; VI: 0/3	2/3; VI: 0/2	1/3; VI: 0/1	1/4; VI: 0/1	1/2; VI: 0/1
Kidney	0/3	0/3	0/3	0/4	0/2
Lung	1/3; VI: 0/1	0/3	0/3	0/4	0/2
Intestine	0/3	0/3	0/3	0/4	0/2
Stomach	0/3	0/3	0/3	0/4	0/2
Rectum	0/3	0/3	0/3	0/4	0/2
Bladder	0/3	0/3	0/3	0/4	0/2
Reproductive organs	0/3	0/3	0/3	0/4	0/2
Salivary glands	0/3	0/3	0/3	0/4	0/2

Abbreviations: ID—identification number; NS—not sampled; PCR—polymerase chain reaction; VI—virus isolation. [a] Data represents the number of positive samples/number tested. Data designates PCR results unless otherwise stated. VI was only attempted on specimens with positive PCR results.

MARV RNA was detected in the spleen of eight seropositive bats from day 3 to 12 p.i. (virus concentration range: $10^{-0.6}$ $TCID_{50}$/g tissue–$10^{2.91}$ $TCID_{50}$/g tissue), and in the liver of six seropositive bats from day 3 to 9 p.i. (virus concentration range: $10^{-0.27}$ $TCID_{50}$/g tissue–$10^{1.75}$ $TCID_{50}$/g tissue).

In comparison, MARV concentrations in the spleens and livers of naive infected bats between days 3 and 12 p.i. ranged from $10^{2.95}$ $TCID_{50}$/g tissue to $10^{3.89}$ $TCID_{50}$/g tissue, and from $10^{2.6}$ $TCID_{50}$/g tissue to $10^{3.7}$ $TCID_{50}$/g tissue, respectively [15]. There were no significant differences between the mean MARV concentrations in the spleens (naive bats: $10^{2.96}$ $TCID_{50}$/g tissue; seropositive bats: $10^{2.57}$ $TCID_{50}$/g tissue; p-value: 0.51) and livers (naive bats: $10^{1.92}$ $TCID_{50}$/g tissue; seropositive bats: $10^{1.5}$ $TCID_{50}$/g tissue; p-value: 0.28) of naive [15] and seropositive bats on day 3 p.i. However, the virus cleared earlier in seropositive bats, with a statistically significant difference in viral loads in the livers (p-value: 0.01) and spleens (p-value: 0.02) of naive and seropositive bats from day 5 p.i.

Viral RNA was detected in the lung of one seropositive bat on day 3 p.i. ($10^{-0.47}$ $TCID_{50}$/g tissue) and in one nasal swab on day 5 p.i. ($10^{0.82}$ $TCID_{50}$/mL) (Table 1). No MARV RNA could be detected in any of the other tissues sampled in our study (Table 1). These findings differ from results obtained from a previous study of experimental MARV infection in naive bats, where MARV RNA could be detected in the salivary glands (18% of bats), kidney (9%), intestine (27%), bladder (5%) and the reproductive tract (18%) between 3 and 12 days p.i. [15]. No MARV RNA was detected in any specimens collected from control bats.

There was a negative correlation ($r_s = -0.61$, Spearman's: $p = 0.001$) between the ELISA PP value and the level of equivalent viremia, suggesting that ERBs are to some extent more likely to become viremic upon reinfection when levels of MARV-specific IgG have declined.

3.4.3. Virus Neutralization Index

The titer of MARV/Ozolin titrated on the SW-13 cells was 1.58×10^7 $TCID_{50}$/mL, and was neutralized to a titer of 1.58×10^6 $TCID_{50}$/mL by the anti-MARV/Matlapitsi antibody positive bat serum. The titer of MARV/Watsa titrated on the SW-13 cells was 3.41×10^6 $TCID_{50}$/mL and was neutralized by the bat serum to a titer of 7.34×10^5 $TCID_{50}$/mL. These results show that the bat serum was somewhat better able to neutralize the MARV/Ozolin strain, although the difference in neutralization was not statistically significant (two-tailed p-value: 0.47).

4. Discussion

While some estimates are available from previous studies [11,15], this study is the first to report on the duration of maternal immunity to MARV in both wild-caught and captive-born juvenile ERBs born to naturally MARV-exposed dams. In wild-caught juveniles, maternal IgG antibodies were present in all bats sampled at approximately 2 months of age, and were undetectable in all but one bat by 5 months of age. Likewise, in captive-born juveniles, maternal antibodies to MARV were undetectable in all bats by 5 months of age. These results show that maternal immunity is lost in juveniles between 4 and 5 months of age, making them susceptible to primary infection with MARV from 5 months of age onwards. The results are consistent with previous estimates of the duration of maternal immunity to MARV by our group [15] and are supported by the results of ecological studies of MARV in ERB populations [11,28]. A longitudinal study by Amman and colleagues [11] showed a peak in MARV infection rates in ERBs of around 6 months of age (MARV RNA detected in 12.4% of 6 month old juvenile ERBs compared to 2.7% in 3 month old ERBs and 0% in pups). In another ecological study, we were also able to demonstrate the presence of MARV RNA in three juvenile bats of at least six months of age, but not in younger juveniles or in adult bats [28]. These observations point to a protective role of maternal antibodies against MARV infection in juvenile bats. The loss of maternal immunity to MARV in juvenile ERBs therefore increases the overall susceptibility of a bat population to MARV infection and may be linked to an increased risk of viral shedding and spillover into the human population. Our results provide a possible time frame representing the period of greatest risk for human infection whilst entering caves inhabited by ERBs.

The duration of the antibody response of experimentally infected ERBs to MARV has previously been investigated by Schuh and colleagues [16]. Their results showed a peak in anti-MARV IgG antibodies at a mean of 20 days p.i. (range: 14–28 days p.i.), corresponding to the period when

viral shedding becomes undetectable [16]. Immunoglobulin G antibodies to MARV then rapidly diminished and became undetectable within 3 months p.i. in both experimentally MARV-infected ERBs, and contact ERBs that had been "naturally" infected by experimentally infected ERBs [16]. In comparison, results from our current study as well as a previous study by our group [15] showed a peak in anti-MARV IgG antibody levels in experimentally infected bats at day 14 p.i. (range: 9–21 days p.i.), with antibodies still being detectable in the majority of the bats (67%) almost 4 months after infection in the current study. It is possible that some bats possess natural genetic differences that allow them to retain longer-term immunity to MARV compared to others, which may explain why some bats in our study rapidly lost immunity to MARV, while others did not.

In contrast to results obtained from experimental inoculation studies, our results in bats naturally exposed to MARV suggest that anti-MARV IgG can persist in the majority of bats at moderately high levels for longer than 11 months. However, because the exposure histories of these bats to MARV are unknown, it is possible that the longer lasting IgG immune response may have resulted from re-exposure to the virus after primary infection had already occurred.

The natural inoculation dose and ports of entry and exit of MARV in ERBs remains unclear. It was recently shown that bats may shed MARV in and possibly become infected through exposure to oral and fecal secretions [14]. Infection in this manner may result in different immune responses in ERBs than infection through sub-cutaneous inoculation, which was the inoculation route chosen for the experimental infection studies discussed in this paper [15,16]. Given the difference in results from studies of antibody-mediated immunity in experimentally and naturally infected ERBs, and possible differing routes and doses of experimental and natural MARV exposure, it is possible that results obtained in experimental infection studies might not accurately reflect what occurs upon natural exposure to MARV.

Further to evaluating the duration of the immune responses of ERBs to MARV, this study has indicated that bats harboring antibodies to MARV can become reinfected upon re-exposure with the virus, albeit with a heterologous isolate. The majority of reinfected bats in our study showed evidence of MARV in the blood, but virus presence in organs was mostly localized to the liver and spleen. This is in contrast to the systemic MARV replication observed in bats with a primary infection [15]. The presence of viremia in seropositive bats after rechallenge, and the isolation of MARV from the serum of one bat on day 3 p.i. indicates the possibility of transmission of MARV via the blood for a short period of time following reinfection.

Replicating virus could not be isolated from any of the qRT-PCR positive tissues tested in our study. Unsuccessful attempts at isolating replicating virus from qRT-PCR positive tissues could possibly be ascribed to the viral load being below the detectable level, inadequate sensitivity of the virus isolation method used, or the presence of immune complexes. It was recently shown that Ebola virus could not be isolated in Vero E6 culture from patient serum samples yielding C*t* values higher than 33.7 [30], and in an ecological study of MARV infection in ERBs in Uganda, MARV could not be isolated in Vero cells from bat tissues with C*t* values higher than 30 [11].

In this study, MARV could not be detected in major tissues that might play a role in viral shedding and transmission in bat populations, such as the salivary glands, intestine, reproductive tract and bladder [14,16]. For this reason, it appears that reinfection of previously exposed bats might not play a major role in the maintenance of MARV in natural bat populations. However, physiological or environmental stress factors such as pregnancy, social stress or poor nutrition may result in enhanced viral replication and shedding when reinfection takes place under such conditions. Furthermore, given the difference in sample size between this study (n = 15) and our previous study (n = 22) [15], it is possible that MARV RNA might have been detected in some of these tissues if a larger number of bats had been included in the current study.

The mechanism by which MARV is able to infect bats that have pre-existing immunity to the virus needs further elucidation. In a previous study, we showed that bats with laboratory induced immunity were able to efficiently control replication of the virus after re-exposure [15]. Similarly,

Schuh et al. were unable to demonstrate viremia or the presence of MARV RNA in tissues sampled from MARV-reinfected bats with an apparent loss of immunity to the virus [31]. The differences in the results obtained from our previous work [15] and the current study could be attributed to the timing of challenge and the status of immunity of the ERBs during re-exposure. Unlike the study conducted by Schuh et al. in which bats had undetectable levels of antibodies to MARV prior to experimental reinfection [31], the bats used in the current study were collected with pre-existing natural immunity to MARV, without knowledge of the period that elapsed between the initial infection and reinfection in the laboratory. In addition, the rechallenge administered in both our previous work [15] and the work by Schuh et al. [31] made use of homologous virus, while the virus used in the current study was heterologous. The challenge virus used in the current study originated from a human patient from the Democratic Republic of the Congo (DRC), whereas the virus circulating in the cave in Matlapitsi Valley is genetically distinct from the DRC isolate, and closely related to the MARV/Ozolin isolate [28]. Several genetically distinct strains and variants of MARV may circulate within a single cave system [10,11,18], and it is possible that antibodies to one variant of MARV do not effectively neutralize variants that are genetically different. In our evaluation of the capability of bat serum positive for anti-MARV/Matlapitsi antibodies to neutralize MARV/Watsa, it was shown that the antibodies were better able to neutralize an isolate closely related to the Matlapitsi variant (MARV/Ozolin) than the Watsa isolate. However, the difference in the reduction of the virus titers was not statistically significant. Whether immunity to one genetic variant of MARV is fully cross-protective against infection with another in ERBs should be explored further.

The T cell responses produced following natural or experimental infection with MARV in ERBs remain to be evaluated. Antibody production does not necessarily correlate with viral clearance in bats [22,23]. In addition, neutralizing antibodies do not always confer protection in vivo, while-non-neutralizing antibodies may confer protection through mechanisms such as antibody-dependent cell-mediated cytotoxicity. The correlates of protection against MARV in ERBs need to be determined.

5. Conclusions

Our results show that passive immunity to MARV is lost in juvenile ERBs between 4 and 5 months of age, making them susceptible to infection with the virus and increasing the risk for spillover into the human population when these bats first become infected with MARV. Exposure to MARV resulted in IgG immune responses lasting at least 110 days p.i. in the majority of experimentally infected bats, and at least 11 months in the majority of naturally infected bats. Our results further suggest that antibodies to MARV in ERBs is likely not completely protective against reinfection. Future research should determine whether a complete loss of IgG antibodies to MARV may again lead to systemic infection and viral shedding in bats inoculated with different MARV isolates and subjected to different stress factors.

Acknowledgments: We thank Gunther Meier and Zibusiso Masuku of the CEZPD-NICD for their assistance with the biosafety aspects of this project. We are also grateful to Alan Kemp for commenting on and proofreading this work, and to Dr Naazneen Moolla for assisting with the graphical abstract. This study was supported by the National Institute for Communicable Diseases, the National Research Foundation of South Africa (86228), the Poliomyelitis Research Foundation (13/54), and the University of Pretoria (postgraduate bursary).

Author Contributions: Janusz T. Paweska, Nadia Storm, Petrus Jansen van Vuren and Wanda Markotter conceived and designed the experiments; Nadia Storm, Janusz T. Paweska and Petrus Jansen van Vuren performed the experiments; Nadia Storm analyzed the data; Nadia Storm wrote the paper, Janusz T. Paweska, Petrus Jansen van Vuren and Wanda Markotter revised the draft paper.

Conflicts of Interest: The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References

1. Sanchez, A.; Geisbert, T.W.; Feldmann, H. *Filoviridae*: Marburg and Ebola viruses. In *Fields Virology*, 5th ed.; Knipe, D.M., Howley, P.M., Eds.; Lippincott Williams & Wilkins: Philadelphia, PA, USA, 2007; pp. 1409–1448. ISBN 0781760607.
2. Conrad, J.L.; Isaacson, M.; Smith, E.B.; Wulff, H.; Crees, M.; Geldenhuys, P.; Johnston, J. Epidemiologic investigation of Marburg virus disease, Southern Africa, 1975. *Am. J. Trop. Med. Hyg.* **1978**, *27*, 1210–1215. [CrossRef] [PubMed]
3. Smith, D.H.; Johnson, B.K.; Isaacson, M.; Swanepoel, R.; Johnson, K.M.; Killey, M.; Bagshawe, A.; Siongok, T.; Keruga, W.K. Marburg-virus disease in Kenya. *Lancet* **1982**, *1*, 816–820. [CrossRef]
4. Johnson, D.; Johnson, B.K.; Silverstein, D.; Tukei, P.; Geisbert, T.W.; Sanchez, A.N.; Jahrling, P.B. Characterization of a new Marburg virus isolated from a 1987 fatal case in Kenya. *Arch. Virol.* **1996**, *11*, 101–114.
5. Bausch, D.G.; Nichol, S.T.; Muyembe-Tamfum, J.J.; Borchert, M.; Rollin, P.E.; Sleurs, H.; Campbell, P.; Tshioko, F.K.; Roth, C.; Colebunders, R.; et al. Marburg hemorrhagic fever associated with multiple genetic lineages of virus. *N. Engl. J. Med.* **2006**, *355*, 909–919. [CrossRef] [PubMed]
6. Centers for Disease Control and Prevention. Imported case of Marburg hemorrhagic fever—Colorado, 2008. *Morb. Mortal. Wkly. Rep.* **2009**, *58*, 1377–1381.
7. Timen, A.; Koopmans, M.P.; Vossen, A.C.; van Doornum, G.J.; Gunther, S.; van den Berkmortel, F.; Verduin, K.M.; Dittrich, S.; Emmerich, P.; Osterhaus, A.D.; et al. Response to imported case of Marburg hemorrhagic fever, the Netherlands. *Emerg. Infect. Dis.* **2009**, *15*, 1171–1175. [CrossRef] [PubMed]
8. Adjemian, J.; Farnon, E.C.; Tschioko, F.; Wamala, J.F.; Byaruhanga, E.; Bwire, G.S.; Kansiime, E.; Kagirita, A.; Ahimbisibwe, S.; Katunguka, F.; et al. Outbreak of Marburg hemorrhagic fever among miners in Kamwenge and Ibanda Districts, Uganda, 2007. *J. Infect. Dis.* **2011**, *204*, S796–S799. [CrossRef] [PubMed]
9. Aceng, J.R. Press Statement on Marburg Virus Disease. Available online: www.mediacentre.go.ug/press-release/press-statement-marburg-virus-disease (accessed on 20 October 2017).
10. Towner, J.S.; Amman, B.R.; Sealy, T.K.; Reeder Carroll, S.A.; Comer, J.A.; Kemp, A.; Swanepoel, R.; Paddock, C.D.; Balinandi, S.; Khristova, M.L.; et al. Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathog.* **2009**, *5*, e1000536. [CrossRef] [PubMed]
11. Amman, B.R.; Carroll, S.A.; Reed, Z.D.; Sealy, T.K.; Balinandi, S.; Swanepoel, R.; Kemp, A.; Erickson, B.R.; Comer, J.A.; Campbell, S.A.; et al. Seasonal pulses of Marburg virus circulation in juvenile *Rousettus aegyptiacus* bats coincide with periods of increased risk of human infection. *PLoS Pathog.* **2012**, *8*, e1002877. [CrossRef] [PubMed]
12. Amman, B.R.; Nyakarahuka, L.; McElroy, A.K.; Dodd, K.A.; Sealy, T.K.; Schuh, A.J.; Shoemaker, T.R.; Balinandi, S.; Atimnedi, P.; Kaboyo, W.; et al. Marburg virus resurgence in Kitaka mine bat population after extermination attempts, Uganda. *Emerg. Infect. Dis.* **2014**, *20*, 1761–1764. [CrossRef] [PubMed]
13. Paweska, J.T.; Jansen van Vuren, P.; Masumu, J.; Leman, P.A.; Grobbelaar, A.A.; Birkhead, M.; Clift, S.; Swanepoel, R.; Kemp, A. Virological and serological findings in *Rousettus aegyptiacus* experimentally inoculated with Vero cells-adapted Hogan strain of Marburg virus. *PLoS ONE* **2012**, *7*, e45479. [CrossRef] [PubMed]
14. Amman, B.R.; Jones, M.E.; Sealy, T.K.; Uebelhoer, L.S.; Schuh, A.J.; Bird, B.H.; Coleman-McCray, J.D.; Martin, B.E.; Nichol, S.T.; Towner, J.S. Oral shedding of Marburg virus in experimentally infected Egyptian fruit bats (*Rousettus aegyptiacus*). *J. Wildl. Dis.* **2015**, *51*, 113–124. [CrossRef] [PubMed]
15. Paweska, J.T.; Jansen van Vuren, P.; Fenton, K.A.; Graves, K.; Grobbelaar, A.A.; Moolla, N.; Leman, P.; Weyer, J.; Storm, N.; McCulloch, S.D.; et al. Lack of Marburg virus transmission from experimentally infected to susceptible in-contact Egyptian fruit bats. *J. Infect. Dis.* **2015**, *212*, S109–S118. [CrossRef] [PubMed]
16. Schuh, A.J.; Amman, B.R.; Jones, M.E.B.; Sealy, T.K.; Uebelhoer, L.S.; Spengler, J.R.; Martin, B.E.; Coleman-McCray, J.D.; Nichol, S.T.; Towner, J.S. Modelling filovirus maintenance in nature by experimental transmission of Marburg virus between Egyptian rousette bats. *Nat. Commun.* **2017**, *8*, 14446. [CrossRef] [PubMed]
17. Swanepoel, R.; Smit, S.B.; Rollin, P.E.; Formenty, P.; Leman, P.A.; Kemp, A.; Burt, F.J.; Grobbelaar, A.A.; Croft, J.; Bausch, D.G.; et al. Studies of reservoir hosts for Marburg virus. *Emerg. Infect. Dis.* **2007**, *13*, 1847–1851. [CrossRef] [PubMed]

18. Towner, J.S.; Pourrut, X.; Albariño, C.G.; Nkoque, C.N.; Bird, B.H.; Grard, G.; Ksiazek, T.G.; Gonzales, J.P.; Nichol, S.T.; Leroy, E.M. Marburg virus infection detected in a common African bat. *PLoS ONE* **2007**, *2*, e764. [CrossRef] [PubMed]
19. Plowright, R.K.; Peel, A.J.; Streicker, D.G.; Gilbert, A.T.; McCallum, H.; Wood, J.; Baker, M.L.; Restif, O. Transmission or within-host dynamics driving pulses of zoonotic viruses in reservoir-host populations. *PLoS Negl. Trop. Dis.* **2016**, *10*, e0004796. [CrossRef] [PubMed]
20. Peel, A.J.; Pulliam, J.R.; Luis, A.D.; Plowright, R.K.; O'Shea, T.J.; Hayman, D.T.; Wood, J.L.; Webb, C.T.; Restif, O. The effect of seasonal birth pulses on pathogen persistence in wild mammal populations. *Proc. Biol. Sci.* **2014**, *281*, 20132962. [CrossRef] [PubMed]
21. Hayman, D.T. Biannual birth pulses allow filoviruses to persist in bat populations. *Proc. Biol. Sci.* **2015**, *282*, 20142591. [CrossRef] [PubMed]
22. Halpin, K.; Hyatt, A.D.; Fogarty, R.; Middleton, D.; Bingham, J.; Epstein, J.H.; Rahman, S.A.; Hughes, T.; Smith, C.; Field, H.E.; et al. Pteropid bats are confirmed as the reservoir hosts of henipaviruses: A comprehensive experimental study of virus transmission. *Am. J. Trop. Med. Hyg.* **2011**, *85*, 946–951. [CrossRef] [PubMed]
23. Middleton, D.J.; Morrissy, C.J.; van der Heide, B.M.; Russell, G.M.; Braun, M.A.; Westbury, H.A.; Halpin, K.; Daniels, P.W. Experimental Nipah virus infection in pteropid bats (*Pteropus poliocephalus*). *J. Comp. Pathol.* **2007**, *136*, 266–272. [CrossRef] [PubMed]
24. Nakayama, E.; Tomabechi, D.; Matsuno, K.; Kishida, N.; Yoshida, R.; Feldmann, H.; Takada, A. Antibody-dependent enhancement of Marburg virus infection. *J. Infect. Dis.* **2011**, *204*, S978–S985. [CrossRef] [PubMed]
25. Brunet-Rossini, A.K.; Wilkinson, G.S. Methods for age estimation and the study of senescence in bats. In *Ecological and Behavioral Methods for the Study of Bats*; Kunz, T.H., Parsons, S., Eds.; The John Hopkins University Press: Baltimore, MD, USA, 2009; pp. 315–325. ISBN 9780801891472.
26. Mutere, F.A. The breeding biology of the fruit bat *Rousettus aegyptiacus E. Geoffroy* living at 0 degrees 22'S. *Acta Trop.* **1968**, *25*, 97–108. [PubMed]
27. Paweska, J.T.; Burt, F.J.; Swanepoel, R. Validation of IgG-sandwich and IgM-capture ELISA for the detection of antibody to Rift Valley fever virus in humans. *J. Virol. Methods* **2005**, *124*, 173–181. [CrossRef] [PubMed]
28. Paweska, J.T.; Jansen van Vuren, P.; McCulloch, S.; Kemp, A.; Storm, N.; Grobbelaar, A.; Scott, T.; Geldenhuys, M.; Mortlock, M.; Moolla, N.; et al. Serological and molecular evidence of Marburg virus infection among *Rousettus aegyptiacus* in South Africa. In Proceedings of the Sixth International Symposium on Filoviruses, Galveston, TX, USA, 30 March–2 April 2014.
29. Hierholzer, J.C.; Killington, R.A. Virus isolation and quantitation. In *Virology Methods Manual*; Mahy, B.W.J., Kangro, H.O., Eds.; Academic Press Inc.: San Diego, CA, USA, 1996; pp. 25–46. ISBN 9780124653306.
30. Jansen van Vuren, P.; Grobbelaar, A.; Storm, N.; Conteh, O.; Konneh, K.; Kamara, A.; Sanne, I.; Paweska, J.T. Comparative evaluation of the diagnostic performance of the prototype Cepheid GeneXpert Ebola assay. *J. Clin. Microbiol.* **2016**, *52*, 35967. [CrossRef] [PubMed]
31. Schuh, A.J.; Amman, B.R.; Sealy, T.K.; Spengler, J.; Nichol, S.T.; Towner, J.S. Egyptian rousette bats maintain long-term protective immunity against Marburg virus infection despite diminished antibody levels. *Sci. Rep.* **2017**, *7*, 8763. [CrossRef] [PubMed]