Production and characterisation of monoclonal antibodies to Ebolavirus nucleoprotein and their application in development of a rapid antigen detection test

By

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Abstract

Viruses belonging to *Filoviridae* family, namely ebolaviruses and marburgviruses, cause filoviral haemorrhagic fever (FHF), resulting in case fatality rates of up to 90 percent depending on the virus species and strain. There has been an increase in the incidence of FHF outbreaks in Africa in the last two decades, with some caused by newly found viruses and others occurring in previously unaffected areas. For early diagnosis of Ebola virus disease (EVD), antigen detection tests are best suited. Ebolavirus nucleoprotein (NP) is ideal for antigen detection because it is highly conserved among ebolaviruses, has strong antigenicity and is abundant in ebolavirus particles. The purpose of this study was to produce and characterise monoclonal antibodies (mAbs) to ebolavirus NP, and with these mAbs develop a specific, sensitive, rapid and easy to use, disposable immunochromatographic strip test for the diagnosis of ebolavirus infections. Plasmids expressing *Zaire ebolavirus* NP, glycoprotein (GP), and viral protein 40 (VP40) were constructed and transfected into 293T cells to produce recombinant NP and virus-like particles (VLPs). The VLPs were inoculated into mice and a panel of mouse mAbs to *Zaire ebolavirus* NP was produced. These mAbs were grouped according to their specificity and cross-reactivity to the other known *Ebolavirus* species. Synthetic peptides were used to map the epitopes recognised by these mAbs, which included two regions highly conserved among the currently known *Ebolavirus* species. A selected mAb, recognising all known *Ebolavirus* species on ELISA and Western blot, was then used to develop an immunochromatography-based rapid diagnostic test. This test was able to positively identify *Zaire ebolavirus*, *Tai Forest ebolavirus* and *Bundibugyo ebolavirus* VLPs. Further studies need to be conducted to optimise the test and determine its specificity and sensitivity to live ebolavirus in comparison to other established diagnostic tests. Once validated, this test has great potential for use in the field as a rapid diagnostic tool for EVD.

Dedication

This work is dedicated to my husband, Dr. Simbarashe Chitanga; my children, Sihle and Thabo; my parents Mr. and Mrs. L.L. Changula; my brothers and sisters and extended family. I appreciate the various roles you have played in my life and your contributions towards the completion of my studies.

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Above all, I want thank my God; through Him all things are possible.

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List of Abbreviations and Acronyms

aa	amino acids
BDBV	Bundibugyo virus
BSL-4	Biosecurity level four laboratory
DRC	Democratic Republic of Congo
DMEM	Dulbeco's modified Eagle's medium
EBOV	Ebola virus
ELISA	Enzyme-linked immunosorbent assay
EVD	Ebola virus disease
FCS	foetal calf serum
ffu	fluorescent focus forming units
FHF	filoviral haemorrhagic fever
GP	glycoprotein
HAT	hypoxanthine-aminopterin-thymidine
HPRGT	hypoxanthine-guanine phosphoribosyl transferase
IFAT	Indirect immunofluorescence antibody test
IFN	interferon

IgG	immunoglobulin G
IgM	immunoglobulin M
kDa	kilo Dalton
LFIA	lateral flow immunoassay
LLOV	Lloviu virus
mAbs	monoclonal antibodies
MARV	Marburg virus
MVD	Marburg virus disease
NP	nucleoprotein
PCR	polymerase chain reaction
RAVV	Ravn virus
RC	Republic of Congo
RESTV	Reston virus
RPMI	Roswell Park Memorial Institute medium
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SUDV	Sudan virus

TAFV	Taï Forest virus
$TCID_{50}$	tissue culture infectious dose 50
TNF	tumour necrosis factor
USA	United States of America
VLP	virus-like particle
VP	viral protein
ZNP	Zaire virus nucleoprotein

CHAPTER ONE

1.0 INTRODUCTION

Viral haemorrhagic fevers (VHF) are caused by RNA viruses belonging to the families *Filoviridae*, *Arenaviridae* and *Bunyaviridae*. Ebola virus disease (EVD) and Marburg virus disease (MVD) are zoonoses (Sanchez *et al*., 2007) whose incidence has been increasing (Bannister, 2010; Feldmann and Geisbert, 2011; Muyembe-Tamfum *et al*., 2012), with some caused by newly found viruses (Le Guenno *et al*., 1995; Towner *et al*., 2008) and some occurring in previously unaffected areas including Guinea, Liberia, Sierra Leone and Nigeria that have suffered the most recent EVD outbreak in 2014 (World Health Organisation, 2014d).

The lack of therapeutics and vaccines for filovirus infections combined with ill-equipped or non-existent diagnostic facilities in the regions of Africa where filoviral haemorrhagic fever (FHF) outbreaks occur; as well as the fact that other pathogens cause clinical symptoms comparable to those of EVD and MVD, highlights the need for rapid, sensitive, reliable and virus-specific diagnostic tests to control the spread of these viruses (Sanchez *et al*., 2007; Lucht *et al*., 2007; Groseth *et al*., 2007; Qiu *et al*., 2011). There is a lack of appropriate field-adapted tests that can be used in areas where outbreaks usually occur, often deep in the rain forest with limited medical, public health, transportation and communication infrastructure, and confirmatory diagnosis is usually delayed, often for several months after the initial cases, resulting in increased secondary transmission (Leroy *et al*., 2000; Burki, 2011; MacNeil *et al*., 2011a). The latest outbreak of EVD in West Africa began in December, 2013, but was only diagnosed in March, 2014 (Baize *et al*., 2014). Most patients present initially with non-specific symptoms like chills, fever, fatigue, headache, myalgia, nausea, vomiting and diarrhoea, and are invariably misdiagnosed with other common illnesses such as malaria,

typhoid fever, shigellosis, meningococcal septicaemia, plague, leptospirosis, anthrax, relapsing fever, typhus, murine typhus, yellow fever, Chikungunya fever and fulminant viral hepatitis. It is often only after failure to respond to anti-malarial and/or antibiotic treatment, followed by illness and/or death of health care providers that filoviral infections are suspected (Groseth *et al*., 2007; Kortepeter *et al*., 2011). Currently there are no licenced vaccines, or treatment available, and control is through isolation of patients and supportive therapy (Jeffs *et al*., 2007; Geisbert and Feldmann, 2011; Roddy *et al*., 2011; Mire *et al*., 2014).

Rapid antigen-detection tests with filovirus-specific monoclonal antibodies (mAbs) are likely one of the best ways for early diagnosis of filovirus infections in the field setting. Ideally the test should have very high sensitivity, as a false negative may result in further propagation of the disease (Leroy *et al*., 2000). Antigen detection is sensitive, and can be used in early disease when viraemia is high and antibodies have not yet developed (Ksiazek *et al*., 1999a). Ebolavirus nucleoprotein (NP) may be the ideal target antigen for detection because of its abundance in filovirus particles and its strong antigenicity (Niikura *et al*., 2001; 2003). It is also highly conserved among *Ebolavirus* species (Wilson and Hart, 2001).

1.1 Statement of the problem

In many ebolavirus outbreaks there is usually a delayed confirmatory diagnosis leading to increased secondary transmission of the disease. This is because: many other diseases exhibit similar symptoms to ebolavirus infections; regions that have recorded large outbreaks are usually in remote areas, deep in the rain forest, with limited medical, public health, transportation and communication infrastructure; and there no diagnostic test that can easily and rapidly detect ebolavirus infections under such conditions. Early diagnosis requires an antigen detection test that is simple, stable and rapid. For diagnosis of ebolavirus infections, detection of ebolavirus NP is ideal because it is highly antigenic, very numerous in virion

particles and is a highly conserved gene. Therefore, there was need for the production and characterisation of mAbs to ebolavirus NP for use in the rapid diagnosis of the disease.

1.2 Objectives

1.2.1 General objectives

To produce mAbs to ebolavirus NP, which can then be used in an immunochromatographic strip test for diagnosis of ebolavirus infections under field conditions.

1.2.2 Specific objectives

1. To produce mAbs to ebolavirus NP.
2. To characterise the mAbs produced.
3. To develop an immunochromatographic strip test to allow for identification of ebolaviruses using these mAbs.

1.3 Significance of the study

Currently, for diagnosis of filovirus infections, enzyme-linked immunosorbent assay (ELISA), serum neutralization test, reverse transcriptase polymerase chain reaction (RT-PCR) assay, electron microscopy and virus isolation by cell culture are used (World Health Organisation, 2014b). These tests are expensive to run, and require specialised training and equipment. Virus isolation can only be conducted in a BSL-4 containment laboratory. Considering that many of the areas in which filovirus outbreaks occur are resource poor, there is a need for a diagnostic test that is simple and rapid to perform that can be used without any special training, storage or handling procedures. This study aims to produce an immunochromatographic strip test that can rapidly detect infection with all known and possible unknown *Ebolavirus* species. Once validated this test could prove a useful tool in the

diagnosis and surveillance of ebolavirus infections, especially in resource poor areas with inadequate or non-existent diagnostic facilities. Ideally it should be able to diagnose all infections caused by any unknown *Ebolavirus* species. The test is expected to be simple to perform; robust with no special storage or handling conditions and rapid, taking only 20 minutes to diagnosis. It will potentially reduce time from first case to confirmatory diagnosis limiting secondary transmission and contribute to better disease outbreak preparedness.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Filoviruses

Ebolaviruses and marburgviruses are single-stranded, negative-sense and non-segmented RNA viruses belonging to the family *Filoviridae*. These filoviruses are known to cause haemorrhagic fever in humans and nonhuman primates (Sanchez *et al*., 2007). Most of the known filoviruses are endemic to Africa, with several different virus species belonging to the genus *Ebolavirus* found in central and western African rain forests, approximately within 10 degrees north and south of the equator (Groseth *et al*., 2007), and the single species belonging to the genus *Marburgvirus* in open dry areas of eastern and south-central Africa (Peterson *et al*., 2004). Filoviruses are among the most lethal human pathogens recognized to date with case fatality rates of up to 90 percent, depending on the virus species and strain (Pittalis *et al*., 2009; Bente *et al*., 2009). Both ebolavirus and marburgvirus are considered to be potential weapons for bio-warfare (Borio *et al*., 2002), and require high containment bio-safety level 4 (BSL-4) laboratories to conduct research on the live virus (Towner *et al*., 2004; Saijo *et al*., 2006b; MacNeil and Rollin, 2012). However, these facilities are only established in a few countries, namely: Canada, France, Germany, Hungary, Italy, Russia, South Africa, Sweden, the United Kingdom and the United States of America (Saijo *et al*., 2006b; Nisii *et al*., 2013).

2.1.1 Filovirus taxonomy

The family *Filoviridae* belongs to the order *Mononegavirales* and contains two genera, *Ebolavirus* and *Marburgvirus*. There is one known species of *Marburgvirus*, namely *Marburg marburgvirus*, consisting of two viruses, Marburg virus (MARV) and Ravn virus (RAVV). In contrast, the genus *Ebolavirus* has five known species, *Zaire ebolavirus*, *Sudan*

ebolavirus, *Taï Forest ebolavirus*, *Bundibugyo ebolavirus* and *Reston ebolavirus*, represented by Ebola virus (EBOV), Sudan virus (SUDV), Taï Forest virus (TAFV), Bundibugyo virus (BDBV) and Reston virus (RESTV), respectively (Fig. 2-1). Furthermore, there is a newly discovered filovirus named Lloviu virus (LLOV) assigned to the proposed genus *Cuevavirus*, with one species, *Lloviu cuevavirus* (Kuhn *et al*., 2010; Negredo *et al*., 2011).

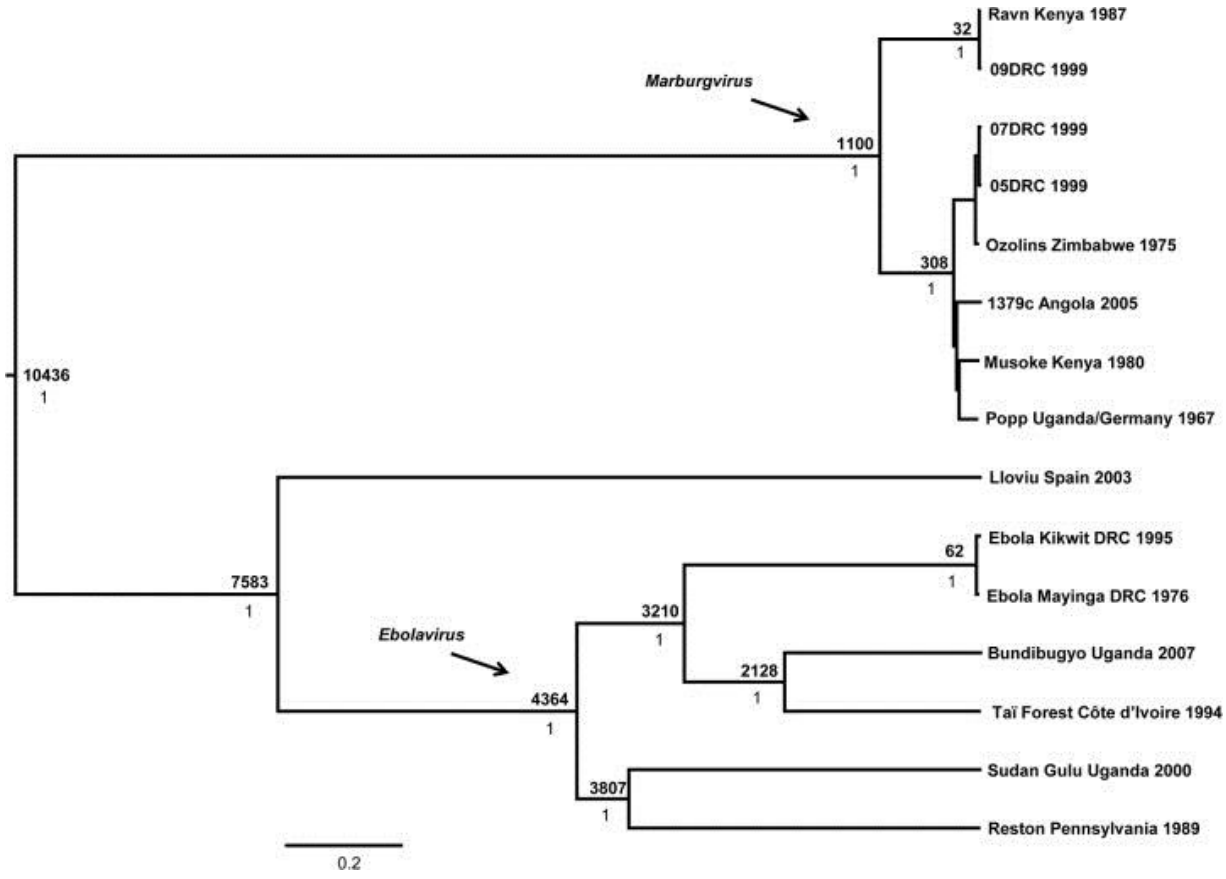

Figure 2-1: Bayesian coalescent analysis of viruses of the family *Filoviridae*. The maximum clade credibility tree is shown with the most common recent ancestor (MRCA), in years before 2007, at each node. Posterior probability values are shown beneath MRCA estimates. Scale is in substitutions/site (Carroll *et al*., 2013).

Based on the comparison of full length genome sequences, the divergence between ebolaviruses and marburgviruses is more than 65 percent (Towner *et al*., 2006). Within ebolaviruses, EBOV differs from TAFV and BDBV by about 37 percent and from SUDV and RESTV by about 42 percent (Towner *et al*., 2008), while MARV and RAVV divergence is

about 21 percent (Towner *et al*., 2006). The nucleotide difference between LLOV and MARV, and LLOV and EBOV are approximately 58 and 52 percent, respectively (Negredo *et al*., 2011).

2.1.2 Filovirus genome

The genome of filoviruses is approximately 19 kb long, composed of linear, non-segmented, negative-sense, single-stranded RNA, and contains seven genes that encode structural and non-structural proteins, arranged sequentially in the order NP, viral protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24 and polymerase (L) genes. However, in ebolaviruses, unlike marburgviruses, mRNA editing of the GP gene results in formation of two forms of protein: a full length envelope, structural GP and a smaller, soluble, non-structural glycoprotein, sGP (Fig. 2-2) (Sanchez *et al*., 2007; Barrette *et al*., 2011). NP, VP35, VP40 and L genes are highly conserved, while GP, VP30 and VP24 are variable (Feldmann and Klenk, 1996).

The envelope GP is a membrane anchored glycoprotein, found on the surface of the virus. It mediates adhesion, entry and internalisation into target cells and is a major determinant of virus pathogenicity (Barrette *et al*., 2011; Bale *et al*., 2012). It also counteracts the host adaptive immune response and antiviral response, decreases the endothelial barrier function and induces production of pro-inflammatory cytokines (de Wit *et al*., 2011). The sGP is more abundant than GP and has been shown to have a potential anti-inflammatory role, having an antagonistic effect to endothelial barrier functions induced by tumour necrosis factor (TNF)-α (Wahl-Jensen *et al*., 2005; Falzarano *et al*., 2006).

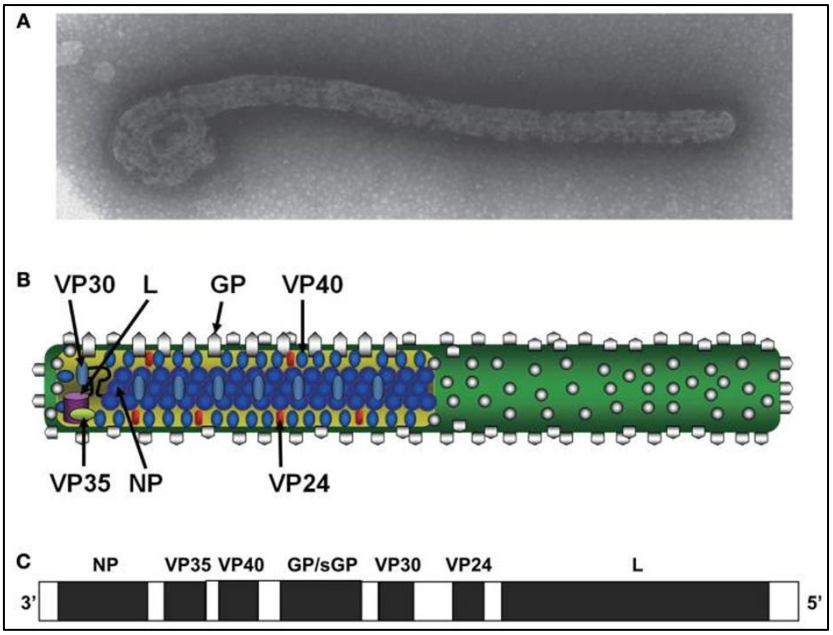

Figure 2-2: Structure of ebolavirus particle and genome organization. (A) Electron micrograph of ebolavirus particle; (B) its schematic diagram and (C) negative-sense genome organization (Takada, 2012).

Transcription and replication of the viral genome are mediated by NP, VP35, VP30 and L genes that are bound to the viral RNA. NP is also involved in encapsidation of the RNA genome, nucleocapsid formation and budding; and is antagonistic to the host immune response. VP35 counteracts the host innate immune response by preventing interferon (IFN)-α/β induced gene expression. VP40 is a matrix protein, involved in particle formation, virus budding and release from host cells. VP24 is also a matrix protein, involved in nucleocapsid formation and assembly, and antagonises the innate immune response by preventing IFN induced cell signalling (Barrette *et al*., 2011; de Wit *et al*., 2011; Brauburger *et al*., 2012).

2.1.3 Ebola virus NP

The average EBOV virion, which is up to 1,028 nm in length, contains about 3,200 NP molecules (Bharat *et al*., 2012). The EBOV NP consists of 739 amino acid (aa) residues, with a conserved hydrophobic N-terminus and a variable hydrophilic C-terminal part (Niikura *et*

al., 2001; Sanchez *et al*., 2007). The NP plays an important role in the replication of the viral genome and is essential for formation of the nucleocapsid (Watanabe *et al*., 2006).

The C-terminus of EBOV NP binds to VP40 while the N-terminus forms a condensed helix with the same diameter as the inner nucleocapsid helix of an EBOV particle (Bharat *et al*., 2012). Following expression of VP40 in cultured cells, virus-like particles (VLPs) are produced and, upon co-expression of NP, the VLP contains NP as its core (Fig. 2-3) (Noda *et al*., 2007; Bharat *et al*., 2012).

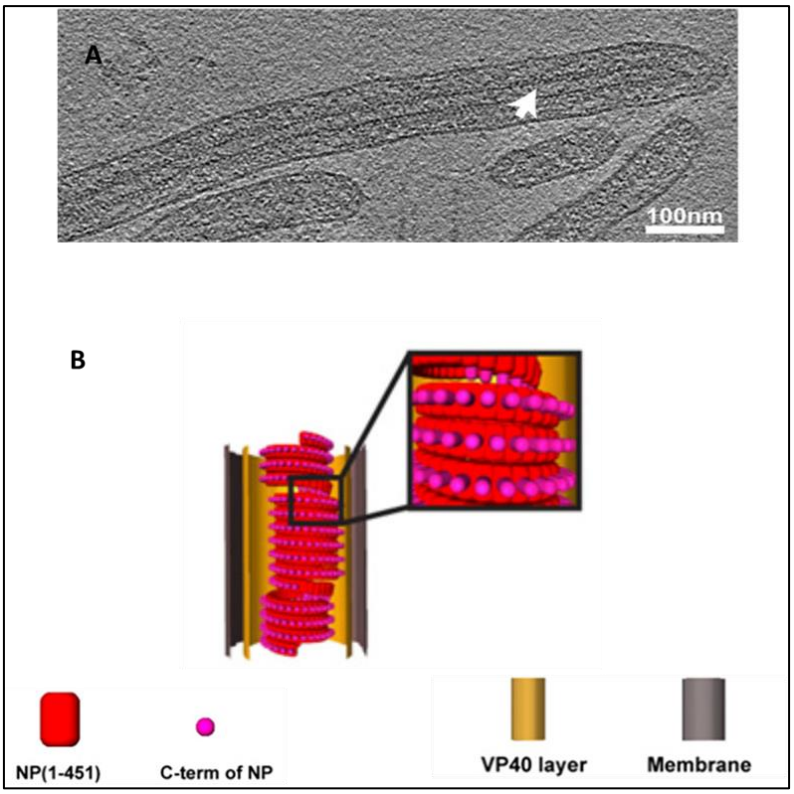

Figure 2-3: Ebolavirus nucleocapsid (A) White arrow head indicates the nucleocapsid within the ebolavirus virion; (B) Schematic illustration of the nucleocapsid helix of VLP (Bharat *et al*., 2012).

It has been demonstrated that the C-terminal half of the filovirus NP has strong antigenicity (Saijo *et al*., 2001a). Multiple studies have identified conformational and linear epitopes for antibodies in this NP region for several viruses within the genus *Ebolavirus* (Niikura *et al*.,

2001; 2003; Ikegami *et al*., 2003a). NP may be the ideal target antigen for detection because of its abundance in filovirus particles and its strong antigenicity (Niikura *et al*., 2001; 2003). It is also highly conserved among *Ebolavirus* species (Wilson and Hart, 2001).

2.2 Filoviral haemorrhagic fevers

Filoviral haemorrhagic fevers are infectious diseases caused but members of the family *Filoviridae*, namely viruses belonging to the genera *Marburgvirus* and *Ebolavirus*. These viruses cause two similar clinical diseases; MVD and EVD respectively (Sanchez *et al*., 2007).

Marburg virus disease was first reported in Marburg, Germany in 1967 (Sanchez *et al*., 2007). This was followed by a few outbreaks of this disease and after 1987 there came a period of quiescence until the Democratic Republic of the Congo (DRC) outbreak of 1994. The first outbreak of EVD was reported in Zaire (now DRC) in 1976 and subsequently Sudan (now South Sudan) had outbreaks in 1976 and 1979 (Leroy *et al*., 2011). This was followed by 15 years of no reported EVD outbreaks in Africa. From 1994, the frequency of outbreaks has increased in Africa, with discoveries of two newly found ebolaviruses in Côte d'Ivoire in 1994 (Formenty *et al*., 1999), and in Uganda in 2007 (Towner *et al*., 2008), now belonging to the species *Taï Forest ebolavirus* and *Bundibugyo ebolavirus*, respectively (Leroy *et al*., 2011). RESTV does not cause disease in humans but can cause disease in nonhuman primates (NHPs) (Sanchez *et al*., 2007). The prevalence and diversity of filoviruses have been shown to be higher than was once thought (Barrette *et al*., 2011). Outbreaks of FHF are increasing (Fig. 2-4) with an increase in the number of people affected (Saijo *et al*., 2006b; Groseth *et al*., 2007; Feldmann and Geisbert, 2011).

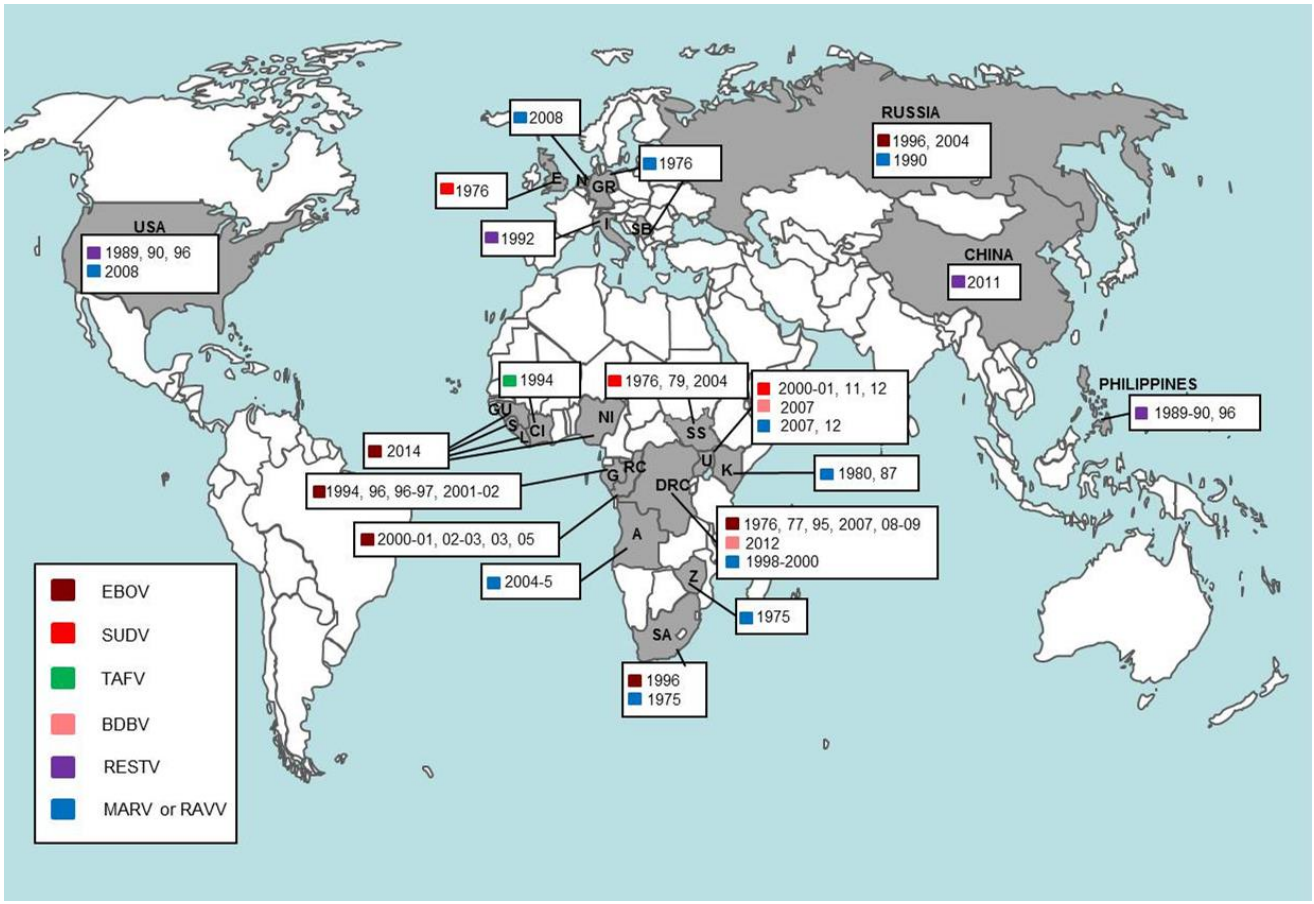

Figure 2-4: Location of FHF outbreaks. Countries that have experienced outbreaks are shaded in grey. Causative filovirus species and year of outbreak are shown in white boxes. EBOV: Ebola virus; SUDV: Sudan virus; TAFV: Tai Forest virus; BDBV: Bundibugyo virus; RESTV: Reston virus; MARV: Marburg virus; RAVV: Ravn virus; A: Angola; CI: Côte d'Ivoire; DRC: Democratic Republic of Congo; E: England, G: Gabon; GR: Germany; GU: Guinea; I: Italy; K: Kenya; L: Liberia; N: Netherlands; NI: Nigeria; RC: Republic of Congo; S: Sierra Leone, SA: South Africa, SB: Serbia; SS: South Sudan; U: Uganda; US: United States; Z: Zimbabwe.

2.3 Epidemiology of filoviruses

2.3.1 Known geographical distribution of filoviruses

Filoviruses are endemic to Africa with most ebolaviruses found in central and western African rain forests, approximately within 10 degrees north and south of the equator (Groseth *et al*., 2007), and marburgviruses in open dry areas of eastern and south central Africa (Peterson *et al*., 2004). The only known geographic sources of RESTV are the Philippines (Miranda *et al*., 2002) and China (Pan *et al*., 2014), while LLOV, a novel ebolavirus-like filovirus, has only been found in bats in Spain (Negredo *et al*., 2011).

2.3.2 Ebola virus disease

Ebola virus disease was first recognized when two large outbreaks occurred in 1976, in South Sudan and the DRC. Large numbers of EVD cases were not reported again until 1994 and 1995, when large outbreaks occurred in Uganda and the DRC, respectively (Sanchez *et al*., 2007). Frequent large outbreaks have since been reported from Gabon, RC, DRC, Uganda (Bannister, 2010), Guinea, Liberia, Sierra Leone and Nigeria (World Health Organisation, 2014d). All outbreaks have occurred within 10 degrees of the equator (Groseth *et al*., 2007). Simultaneously, with these human outbreaks, major EVD outbreaks also affected animal species, including gorillas, chimpanzees and duikers, apparently accounting for the sharp animal population declines observed in these regions (Pourrut *et al*., 2005).

There has been an increase in EVD outbreaks in Africa, probably a result of increased contact of humans and wildlife due to extensive deforestation, hunting and mining (Muyembe-Tamfum *et al*., 2012). The *Ebolavirus* species have a complete genome sequence divergence of 37 - 42 percent (Towner *et al*., 2008). The case fatality rates (CFRs) of the different ebolaviruses causing these EVD outbreaks also vary (Table 2-1).

Table 2-1: Outbreaks of Ebola virus disease

Year	Location	Virus	Human Cases	Deaths	CFR (%)[†]
1976	Democratic Republic of Congo (formerly Zaire)	EBOV	318	280	88
1976	South Sudan (formerly Sudan)	SUDV	284	151	53
1976	England	SUDV	1*	0	0
1977	Democratic Republic of Congo	EBOV	1	1	100
1979	South Sudan	SUDV	34	22	65
1989	USA	RESTV	0[‡]	0	0
1990	USA	RESTV	4[‡]	0	0
1989-1990	Philippines	RESTV	3[‡]	0	0
1992	Italy	RESTV	0[‡]	0	0
1994	Gabon	EBOV	52	31	60
1994	Côte d'Ivoire	TAFV	1	0	0
1995	Democratic Republic of Congo	EBOV	315	250	79
1996	Gabon	EBOV	37	21	57
1996-1997	Gabon	EBOV	60	45	75
1996	South Africa	EBOV	2 [§]	1	50
1996	USA	RESTV	0[‡]	0	0
1996	Philippines	RESTV	0[‡]	0	0
1996	Russia	EBOV	1*	1	100
2000-2001	Uganda	SUDV	425	224	53
2001-2002	Gabon	EBOV	65	53	82

Year	Location	Virus	Human Cases	Deaths	CFR (%)[†]
2001-2002	Republic of Congo	EBOV	57	43	75
2002-2003	Republic of Congo	EBOV	143	128	89
2003	Republic of Congo	EBOV	35	29	83
2004	South Sudan	SUDV	17	7	41
2004	Russia	EBOV	1*	1	100
2005	Republic of Congo [(a)]	EBOV	12	10	83
2007	Democratic Republic of Congo	EBOV	264	187	71
2007-2008	Uganda	BDBV	149	37	25
2008	Philippines	RESTV	6[¶]	0	0
2008-2009	Democratic Republic of Congo	EBOV	32	15	47
2011	China [(b)]	RESTV	X[¶¶]		
2011	Uganda	SUDV	1	1	100
2012	Uganda	SUDV	11	4	36
2012	Democratic Republic of Congo	Bundibugyo	36	13	36
2012	Uganda	SUDV	6	3	50
2014**	Guinea [(c)]	EBOV	495	367	74
2014**	Liberia [(c),]	EBOV	554	294	53
2014**	Sierra Leone [(c)]	EBOV	717	298	42
2014**	Nigeria [(c)]	EBOV	13	2	15

†CFR: case fatality rate; *: Laboratory contamination; ‡: Non-human primates infected, asymptomatic humans, infection originated from monkeys from the Philippines; § Index patient came from Gabon; ¶: First report of *Reston ebolavirus* in pigs, asymptomatic humans; ¶¶: *Reston ebolavirus* in pigs, humans not tested; **: Situation as of 8th August 2014,

outbreak still on-going, includes laboratory-confirmed, probable and suspected cases. Table adapted from Centers for Disease Control and Prevention, 2014a apart from (a): World Health Organisation, 2014b; (b): Pan *et al*., 2014 and (c): World Health Organisation, 2014d.

2.3.2.1 Ebola virus

Outbreaks caused by EBOV, representing the species *Zaire ebolavirus* can cause sporadic infections in humans, usually resulting in self-limiting outbreaks (Jezek *et al*., 1999). The genetic diversity between EBOV strains so far isolated is low (Carroll *et al*., 2013). For instance two separate outbreaks caused by EBOV occurred in Luebo, DRC in 2007 and 2008, and the sequences of the viruses in both outbreaks were almost identical and related to previously isolated strains including the one causing the first reported outbreak in Yambuku, DRC in 1976 (Grard *et al*., 2011). Most recently, there was an outbreak of haemorrhagic fever caused by EBOV in the west African countries of Guinea, Liberia and Sierra Leone; and full genome sequencing of virus isolated from three cases has confirmed the virus to have 97 percent homology to DRC and Gabon strains of EBOV (Baize *et al*., 2014). While TAFV, an ebolavirus belonging to a different species (i.e. *Taï Forest ebolavirus*) has been found in the Taï Forest, Côte d'Ivoire (Formenty *et al*., 1999), the 2014 outbreak in west Africa is the first ever reported incidence of EBOV infection in this region (Gatherer, 2014).

2.3.2.2 Sudan virus

As opposed to EBOV, SUDV representing the species *Sudan ebolavirus* was much more confined geographically with all the past outbreaks occurring in a 400 mile range. Genetic diversity between the different SUDV strains is very low (Carroll *et al*., 2013). In 2011, seven years after its last appearance, there was a fatal case of SUDV infection in Uganda, and the full-length genome sequence of the isolate showed 99.3 percent identity to the one that

caused the Gulu outbreak in 2000 (Shoemaker *et al*., 2012). There were two distinct outbreaks caused by SUDV in Uganda in 2012 with independent chains of transmission (Albarino *et al*., 2013) (Table 2-1). In each of the outbreaks there was high sequence identity between the strains isolated within the individual outbreaks. The strain causing the outbreak in November of the same year had the closest sequence identity to the Gulu 2000 outbreak strain (Albarino *et al*., 2013).

2.3.2.3 Tai Forest virus

Tai Forest virus, representing the species *Tai Forest ebolavirus***,** was discovered in 1994 in Côte d'Ivoire after an outbreak of haemorrhagic fever in chimpanzees resulted in non-fatal human infection in during a chimpanzee autopsy (Le Guenno *et al*., 1995). This was the first case of EVD in west Africa (Sanchez *et al*., 2007).

2.3.2.4 Bundibugyo virus

The first recorded outbreak caused by BDBV representing the species *Bundibugyo ebolavirus* occurred in Uganda in 2007 (Towner *et al*., 2008) (Table 2-1). The virus was found again in the 2012 outbreak in Isiro, DRC, which was the first emergence of BDBV in DRC. The BDBV isolate showed 98.6 percent full genome sequence identity with the prototype BDBV isolated in the 2007 outbreak in Bundibugyo, Uganda (Albarino *et al*., 2013).

2.3.2.5 Reston virus

The RESTV, representing the species *Reston ebolavirus*, was first discovered in 1989, during an outbreak of the disease in the USA in Cynologus macaques imported from the Philippines (Jahrling *et al*., 1990). Other outbreaks in monkeys occurred in research facilities in 1990 (USA), 1992 (Italy) and 1996 (USA) (Morikawa *et al*., 2007). There is no evidence of disease in humans infected with RESTV, though pig farm workers and swine product handlers were

found to have RESTV IgG antibodies following infection in pigs. It has been suggested that passage of the virus in pigs may result in a change in its pathogenicity (Barrette *et al*., 2009).

2.3.3 Marburg virus disease

Marburg virus disease was discovered in 1967, originating from infected monkeys imported from Uganda into Germany and Yugoslavia (now Serbia) (Bannister, 2010). The first case of MVD in Africa was reported in 1975; a tourist who visited Zimbabwe developed haemorrhagic fever in South Africa (Conrad *et al*., 1978; Leroy *et al*., 2011). There were a few outbreaks of this disease and after 1987 there came a period of quiescence until the DRC outbreak in 1998. Some incidences of this disease may probably be unreported, as investigators of the 1998-2000 Durba outbreak discovered that there had been unreported incidences of a haemorrhagic syndrome since at least 1987 (Brauburger *et al*., 2012).

The latest MVD outbreaks have occurred in Uganda in 2012 (Table 2-2). Marburgvirus infections in the Egyptian fruit bats have been found to have seasonal fluctuations, with biannual peaks, which correspond to infections in humans (Amman *et al*., 2012). The 2012 outbreak occurred during one of the peaks of marburgvirus infections in bats. The full length genome sequences from that outbreak showed 99.3 percent to marburgviruses found from captured bats in 2008 and 2009 in a nearby cave (Albarino *et al*., 2013).

In 2007 there were two independent outbreaks in Uganda, occurring in miners that had close contact with bats. In June 2007, three people were infected and one died, while in the later outbreak there was only one case and no mortality (Adjemian *et al*., 2011). There was 21 percent sequence variation found between the full-length RNA genome of these viruses, with the earlier one closely related to historical MARV sequences, and the later one more related to RAVV that was first isolated in Kenya in 1987. Both MARV and RAVV-related

sequences were also found in the fruit bats (*Rousettus aegyptiacus*) in the same area (Towner *et al*., 2009).

The 2004-2005 MVD outbreak in Angola was the first report of MVD outside east Africa. There was very little genome sequence variation between the viruses isolated during the outbreak, with some being 100 percent identical. Despite the large geographic distance between Angola and the other known locations which experienced MVD, phylogenetic analysis using the complete viral genome sequences put Angolan strains within the same clade as the majority of east African isolates (Towner *et al*., 2006).

Whereas CFRs for MVD are variable (Table 2-2), MARV-Angola strain is thought to be more pathogenic than other MARV strains such as the Musoke strain (Geisbert *et al*., 2007; Matsuno *et al*., 2010; Nakayama *et al*., 2011).

Table 2-2: Outbreaks of Marburg virus disease

Year	**Country**	**Virus strain**	**Cases**	**Deaths**	**CFR (%)**[†]
1967	Germany and Serbia (formerly Yugoslavia)	MARV[*]	31[‡]	7	23
1975	South Africa (from Zimbabwe)	MARV	3[§]	1	33
1980	Kenya	MARV	2	1	50
1987	Kenya	RAVV[¶]	1	1	100
1990	Russia	Not confirmed	1	1	100
1998-2000	Democratic Republic of Congo	MARV RAVV	154	128	83

Year	Country	Virus strain	Cases	Deaths	CFR (%)[†]
2004-2005	Angola	MARV	252	227	90
2007	Uganda [(a, b)]	MARV	3	1	33
		RAVV	1	0	0
2008	USA (from Uganda)	MARV	1[§]	0	0
2008	Netherlands (from Uganda)	MARV	1[§]	1	100
2012	Uganda [(c)]	MARV	15	4	27

†CFR: case fatality rate; *MARV: Marburg virus; ‡: Infection from African green monkeys from Uganda; §: Index case was tourist; ¶ RAVV: Ravn virus. Table adapted from Brauburger *et al*., 2012 and Centers for Disease Control and Prevention, 2014b, except for (a): Adjemian *et al*., 2011; (b): Towner *et al*., 2009 and (c): Albarino *et al*., 2013.

2.3.4 Reservoir hosts

The *Rousettus aegyptiacus* bats have been identified as a natural reservoir host for *Marburgvirus*, with cyclical fluctuations of infection, which correspond to infections in humans (Amman *et al*., 2012). Many of the outbreaks have been associated with entry into active/decommissioned mines or caves (Peterson *et al*., 2006) where the bats roost.

The *Rousettus aegyptiacus* is the only bat species from which infectious marburgviruses have been isolated. However, filovirus genome RNAs have also been detected in *Epomops franqueti*, *Hypsignathus monstrosus* and *Myonycteris torquata* for EBOV (Leroy *et al*., 2005), *Miniopterus inflatus* and *Rhinolophus eloquens* for marburgviruses (Swanepoel *et al*., 2007), and *Miniopterus schreibersii* for LLOV (Negredo *et al*., 2011). Furthermore, antibodies to various filoviruses have been found in several bat species: anti-EBOV antibodies in *Eidolon helvum*, *Epomophorus gambianus*, *Micropteropus pusillus*, *Tadarida*

condylura and *Rousettus leschenaultii* (Pourrut *et al*., 2009; Hayman *et al*., 2010; 2012; Olival *et al*., 2013); anti-RESTV antibodies in *Cynopterus sphinx, Hipposideros pomona, Miniopterus schreibersii, Myotis pilosus, Pipistrellus pipistrellus, Rousettus amplexicaudatus* and *Rousettus leschenaultii* (Taniguchi *et al*., 2011; Yuan *et al*., 2012; Olival *et al*., 2013) and anti-marburgvirus antibodies in *Hypsignathus monstrosus* and *Epomops franqueti* (Pourrut *et al*., 2009). Currently there are no reports of bats with evidence of exposure to SUDV, TAFV and BDBV (Olival and Hayman, 2014).

It has been suggested that ebolavirus circulation may be very complex, involving far more than simple direct passage from the reservoir to susceptible hosts. It is also possible that there are several reservoir species, and that many other animal species are susceptible to the virus and thereby participate in the natural ebolavirus life cycle (Pourrut *et al*., 2005). The role of potential amplifying hosts has also been suggested (Feldmann and Geisbert, 2011).

2.3.5 Susceptible animals

The NHPs and duikers are affected by EBOV, with a large decline in NHP populations just before and during some human outbreaks (Leroy *et al*., 2004a; Leroy *et al*., 2004b). Between 2002 and 2003, EBOV caused 90-95 percent mortality rates in NHPs in RC, with about 5000 animals dying in the Lossi Sanctuary alone (Bermejo *et al*., 2006). An outbreak of EBOV in Odzala-Kokoua National Park in the RC between 2003 and 2004 resulted in deaths of 95 percent of the gorillas, with higher mortalities in the females and young individuals (Caillaud *et al*., 2006). Higher mortalities are more likely to occur in females and their young probably due to their social interactions.

Dogs can be asymptomatically infected after eating infected carcasses, or licking body fluids from infected patients and may also potentially transmit infections (Allela *et al*., 2005). In the

Philippines and China, domestic swine have been shown to have concurrent infections of porcine reproductive and respiratory disease and RESTV infection (Barrette *et al*., 2009; Pan *et al*., 2014). Experimental infection of pigs with EBOV resulted in a respiratory syndrome, with pigs actively shedding virus and transmitting it to naïve pigs (Kobinger *et al*., 2011). It has been suggested that pigs could be accidental hosts and transmit RESTV or even EBOV to humans (Bausch, 2011).

2.3.6 Transmission and spread in humans

In most of the past FHF outbreaks, it was observed that there are usually one or a few primary introductions of infection to humans which are subsequently spread by human-to-human transmission (Bausch *et al*., 2006; MacNeil *et al*., 2011a). There were however, multiple, short, independent chains of human-to-human transmission in the 1998 MVD outbreak in DRC with at least nine genetic lineages of virus involved, and multiple independent chains of transmission from infected NHPs in the 2001 EVD outbreaks in Gabon and RC (Leroy *et al*., 2004a; Bausch *et al*., 2006). Some of the outbreaks of EVD are thought to be associated with hunting and processing of bush meat, while MVD outbreaks have often been associated with entry into caves and active/decommissioned mines (Leroy *et al*., 2004a; Bausch *et al*., 2006; Adjemian *et al*., 2011).

Primary infection is followed by human-to-human transmission from contact with body fluids of infected individuals (MacNeil *et al*., 2011a; Brauburger *et al*., 2012). The EBOV can be shed in a variety of bodily fluids including saliva, breast milk, stool, and tears during the acute phase of illness. There is a risk of EBOV being transmitted to others during the convalescent phase via breast milk and semen, as the viruses have been found in breast milk and semen up to 15 and 40 days post infection, respectively (Bausch *et al*., 2007), while another study found EBOV RNA in semen 91 days after onset of illness (Rowe *et al*., 1999).

MARV has also been isolated from semen and linked conclusively to sexual transmission 13 weeks into convalescence (Bausch *et al*., 2006).

In a large number of cases, hospital settings are involved in transmission of these diseases to both medical personnel and the general population due to overcrowding, re-use of unsterilized needles and lack of personal protective equipment for barrier nursing. Ignorance of the disease also contributes to increased transmission, as some attribute the cause to evil spirits, and even deliberate infection by foreign health care providers. In many instances probable cases are concealed to avoid social stigma in the community, while others are in denial and prefer to treat their sick relatives at home (Baron *et al*., 1983; Khan *et al*., 1999; Bausch *et al*., 2006; MacNeil *et al*., 2011a; Promed-mail, 2014; World Health Organisation, 2014a). Infected individuals also pass on the infection to family members and other close contacts within the community, especially caregivers. Infection can also be transmitted through traditional funeral rites such as preparation of the body for burial (Bausch *et al*., 2003; Feldmann and Geisbert, 2011).

Following the 2008 outbreak of RESTV in swine in the Philippines, some individuals who worked on pig farms or with swine products had positive serum immunoglobulin G (IgG) titres to RESTV, confirming the potential transmission from pigs to humans (Barrette *et al*., 2009).

There is usually a delay between the initial cases and diagnosis of FHF. This is due to the remoteness of most areas affected, the ill equipped medical facilities and the fact that signs and symptoms of FHF are mainly non-specific, leading them to be misdiagnosed as other more frequent infections that are endemic to the area (MacNeil *et al*., 2011a; MacNeil and Rollin, 2012). While it is possible that neglected cases occur without virus-specific laboratory

diagnosis, outbreaks of FHF have indeed been increasingly reported (Bannister, 2010; Feldmann and Geisbert, 2011; Muyembe-Tamfum *et al*., 2012).

2.3.7 Pathology and pathogenesis of Filovirus haemorrhagic fevers

Incubation period of FHF is variable, between three to 21 days, typically about a week. It starts as a non-specific illness with flu-like symptoms including fever, malaise, loss of appetite, muscle pains and headache, lasting a variable number of days. During the first week of illness, viraemia increases and in survivors, the virus levels in the blood falls to undetectable levels by the end of the second week of illness. In fatal cases viraemia remains elevated until death. The disease rapidly progresses to an intense cytokine response, with severe inflammatory tissue damage. There may be abdominal pain, nausea, diarrhoea, vomiting, sore throat, cough, and arthralgia, with the development of a maculopapular rash. Lymphadenopathy, leukopaenia and thrombocytopaenia also occur. There is also a failure of effective immune responses, so that neither viral replication nor the inflammatory process can be controlled. This results in an overwhelming sepsis syndrome and endothelial damage (Fig. 2-5). The patient may exhibit neurological symptoms, dyspnoea, increased vascular permeability and oedema. Many patients will exhibit a haemorrhagic syndrome, including melena, haematemesis, petechiae, epistaxis, bleeding from the gums, venepuncture sites and conjunctiva. Direct viral invasion of endothelial cells can also cause cell detachment and death, with a major failure of vascular integrity. Death occurs eight – 16 days after onset of symptoms due to shock and multi-organ failure. In fatal cases, there is high production of pro-inflammatory cytokines, inhibition of IFN type 1 response, apoptosis of T-lymphocytes and no antibody response. Survivors have detectable levels of IgM within the first week of illness which peaks in the second week, while IgG can be detected soon after IgM appears, and can be detected for years. Individuals that survive undergo prolonged convalescence

(Bannister, 2010; Warfield and Olinger, 2011; de Wit *et al*., 2011; Kortepeter *et al*., 2011; Brauburger *et al*., 2012).

It must be noted here that not all patients will exhibit haemorrhagic manifestations, hence the change of name from ebola haemorrhagic fever and marburg haemorrhagic fever, to ebola virus disease and marburg virus disease, respectively (Kortepeter *et al*., 2011; Baize *et al*., 2014).

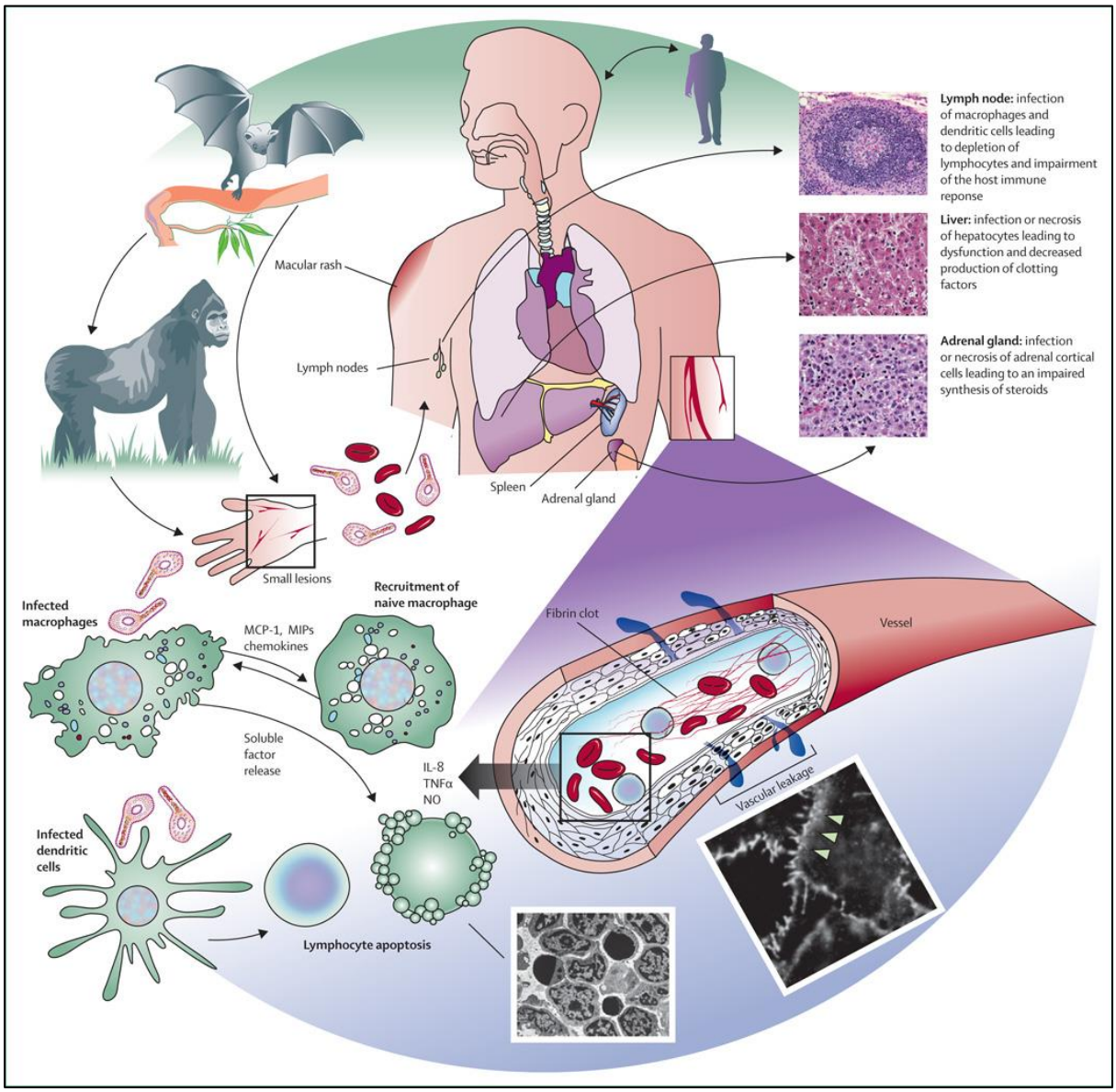

Figure 2-5: Model of Ebola virus pathogenesis. Virus spreads from the initial infection site (small lesions) to regional lymph nodes, liver, and spleen. The systemic virus spread and

replication, the general dysregulation of the host immune response, the coagulation abnormalities, the impairment of the vascular system, and hypotension all together finally result in shock and multi-organ failure. IL: interleukin, MCP-1: monocyte chemo-attractant protein-1, MIPs: macrophage inflammatory proteins, NO: nitric oxide and TNFα: tumour necrosis factor α (Feldmann and Geisbert, 2011).

2.3.8 Potential for outbreaks in previously uninfected African countries

While filovirus outbreaks have been reported in very few countries in Africa (Tables 2-1 and 2-2), there is a potential for outbreaks to occur in previously uninfected areas. Several factors may increase the opportunity for the spread of the disease from one country to the other.

2.3.8.1 Potential role of bats for filovirus transmission

In general, bats and many potential pathogens are thought to have co-evolved and circulated for thousands of years, and recently there has been increased spill over of zoonotic pathogens to humans. Human encroachments into previously uninhabited areas are a contributing factor (Wibbelt *et al*., 2010; Smith and Wang, 2013).

The *Eidolon helvum* is a straw coloured migratory fruit bat with its primary habitat located in equatorial Africa. It is found in large colonies in Angola, Ivory Coast, Malawi, Mauritania, Nigeria, Uganda and Zambia (Richter and Cumming, 2006) often roosting in trees within towns as well as islands on rivers or lakes (Racey, 2004). Between mid-October and late December each year, major *Eidolon helvum* colonies, consisting of five to ten million bats, congregate in the Central Province of Zambia (Richter and Cumming, 2006). Some bat colonies have been shown to migrate more than 2500 km (Richter and Cumming, 2008). While ebolavirus has never been isolated from these bats, antibodies specific to EBOV have been detected (Hayman *et al*., 2010). If these bats shed the infectious virus, they may have

the potential to transmit ebolavirus infection between their primary habitats and their migratory sites, putting a large part of sub-Saharan Africa at risk of infection with ebolavirus.

Filovirus ecology is not yet well understood. While bats may play an important role in filovirus transmission (Olival and Hayman, 2014), there may be other animal species involved, including pigs (Barrette *et al*., 2009), dogs (Allela *et al*., 2005), duikers (Leroy *et al*., 2004a) and NHPs (Leroy *et al*., 2004a; 2004b). Although the effects of climate change on infectious diseases are poorly understood, it likely affects wildlife habitats and densities, which may have the potential to increase the frequency of disease outbreaks by increased risk of exposure of humans to the reservoir hosts and/or increased viral load in these hosts affecting the likelihood of transmission of infection (Mills *et al*., 2010).

2.3.8.2 Geographical distribution of filoviruses

While FHF outbreaks have been reported in limited countries in Africa (Fig. 2-4), geographical distribution of filoviruses may be wider than previously thought. A feature of recent outbreaks is new strains/species in new locations, as has been the case with the MVD outbreak in Angola, the discovery of BDBV in Uganda and DRC, and the current EBOV infection in west Africa (Towner *et al*., 2006; Towner *et al*., 2008; Albarino *et al*., 2013; Baize *et al*., 2014). Using ecological niche modelling, filovirus distribution was generally predicted to occur across the Afro-tropics, with ebolaviruses occurring in the central and western African rain forests and marburgviruses in the drier and less forested central and eastern Africa (Peterson *et al*., 2004). Countries like Zambia, Tanzania, Mozambique, Madagascar and Mauritania have had no reported outbreaks of filovirus infections, but do fall within this ecological niche for the virus and its reservoir(s). There is indeed the possibility of misdiagnosed and undiagnosed cases in countries with no FHF outbreak history. In some areas with no recorded outbreak of EVD, EBOV seroprevalence in humans and some species

of nonhuman primates has been found to be unexpectedly high (Leroy *et al*., 2004b; Becquart *et al*., 2010). This suggests either the presence of non-pathogenic variants of EBOV or unknown filoviruses virus antigenically similar to EBOV, but with lower pathogenicity causing a high seropositivity (Leroy *et al*., 2004b; Gonzalez *et al*., 2005; Lahm *et al*., 2007; MacNeil *et al*., 2011b). This also implies high exposure of these populations to the virus (Becquart *et al*., 2010). Wider filovirus distribution even into the Eurasian continent has been suggested by recent studies: the discovery of RESTV in domestic pigs in China (Pan *et al*., 2014); identification of a new filovirus, LLOV in Spain (Negredo *et al*., 2011) and detection of antibodies to filoviruses or unknown filovirus-related viruses in Indonesian orangutans (Nidom *et al*., 2012) and fruit bats in Bangladesh (Olival *et al*., 2013).

2.3.8.3 Possible anthropogenic factors contributing to frequent outbreaks of FHF in African countries

An increasing population with an increasing demand for resources has forced people to intrude into previously uninhabited land, for agriculture and mining activities, potentially bringing humans into contact with unknown pathogens, reservoir hosts and/or amplifying hosts (Bannister, 2010; Cascio *et al*., 2011). Wildlife trade, much of which is conducted informally and/or illegally, can also increase the risk of outbreaks. Contact of hunters, middle-men and consumers with wildlife can increase the possibility of disease transmission from infected animals (Karesh *et al*., 2005). There has been an association between hunting, butchering and consuming infected carcasses and outbreaks of EVD (Leroy *et al*., 2004a; MacNeil *et al*., 2011b). The only recorded human case of TAFV was in a researcher who contracted the infection during autopsy of chimpanzees (Le Guenno *et al*., 1995). The source of infection in the 2007 outbreak of EVD in DRC is reported to have been traced back to freshly killed bats bought for consumption (Leroy *et al*., 2009). Index cases in the 2001 EVD

outbreaks in Gabon and RC acquired the infection from handling animal carcasses (Leroy *et al*., 2004a).

There is also a risk of exposure of human populations to infected people and/or animals due to the increase in travel (Bannister, 2010; Brown, 2010). On several occasions MARV was imported by tourists; from Zimbabwe to South Africa in 1975 and from Uganda to the USA and the Netherlands in 2008 (Centers for Disease Control and Prevention, 2014b). The EBOV was also imported into South Africa by a medical practitioner from Gabon in 1996 (Centers for Disease Control and Prevention, 2014a). In the most recent outbreak of EVD in west Africa, the disease was first reported in southern Guinea forests followed by a spread into other districts as well as the capital city, Conakry (Gatherer, 2014). The disease was also spread to Liberia from individuals that had a recent history of travel to Guinea, while two suspected cases died in Guinea and were repatriated to Sierra Leone for burial (World Health Organisation, 2014c).

During outbreaks, several factors increase the risk of further spread of the disease. Areas experiencing outbreaks are usually resource poor, and have severely constrained health services coupled with lack of personal protective equipment and inadequate medical health personnel, who also lack knowledge of the disease, especially risk factors for infection (MacNeil *et al*., 2011a; The Lancet, 2014). Ignorance in the communities affected also plays a large role in the further transmission of the disease, and in the recent west African outbreak, there have been reports of communities in denial, with some people believing the disease was caused by the devil, or was brought in by politicians and even foreign medical personnel, resulting in infected individuals and their families not wanting to receive medical attention (The Lancet, 2014; Promed-mail, 2014; World Health Organisation, 2014a).

Though there has been no recorded outbreaks of filovirus infection due to displacement of people from areas of war and civil strife, there is the potential for transmission of diseases to new areas in such situations (Cascio *et al*., 2011), as in the case of increased risk of re-emergence of lymphatic filariasis in Thailand from Burmese refugees (Beyrer *et al*., 2007; Ramaiah, 2013). There are currently over 2.6 million internally displaced people in DRC and over 450,000 refugees in neighbouring countries (UNHCR, 2014a). Inter-ethnic conflict in South Sudan has resulted in a large number of internally displaced people as well as refugees. South Sudan also hosts refugees from other countries, including DRC (UNHCR, 2014b).

2.4 Diagnosis

The control of FHF outbreaks relies on detection of cases, therefore it is important that all potentially infectious individuals be identified (MacNeil *et al*., 2011a). Definitive diagnosis for filoviruses can be obtained through enzyme-linked immunosorbent assay (ELISA), serum neutralization test, reverse transcriptase polymerase chain reaction (RT-PCR) assay, electron microscopy and virus isolation by cell culture (World Health Organisation, 2014b). Identification of the causative agent in the first filovirus disease outbreak in 1967 was through electron microscopy (Fig. 2-6) (Brauburger *et al*., 2012). Currently, the primary assays for diagnosis of filovirus infection are RT-PCR and antigen detection ELISA (Feldmann and Geisbert, 2011). A collection of tests to make a definitive diagnosis is preferred to using just one due to the potentially serious effects of having a false negative or a false positive (Towner *et al*., 2004). It is important to improve the capacity to do filovirus diagnostic testing within affected countries so that outbreak measures can be implemented rapidly (MacNeil *et al*., 2011a).

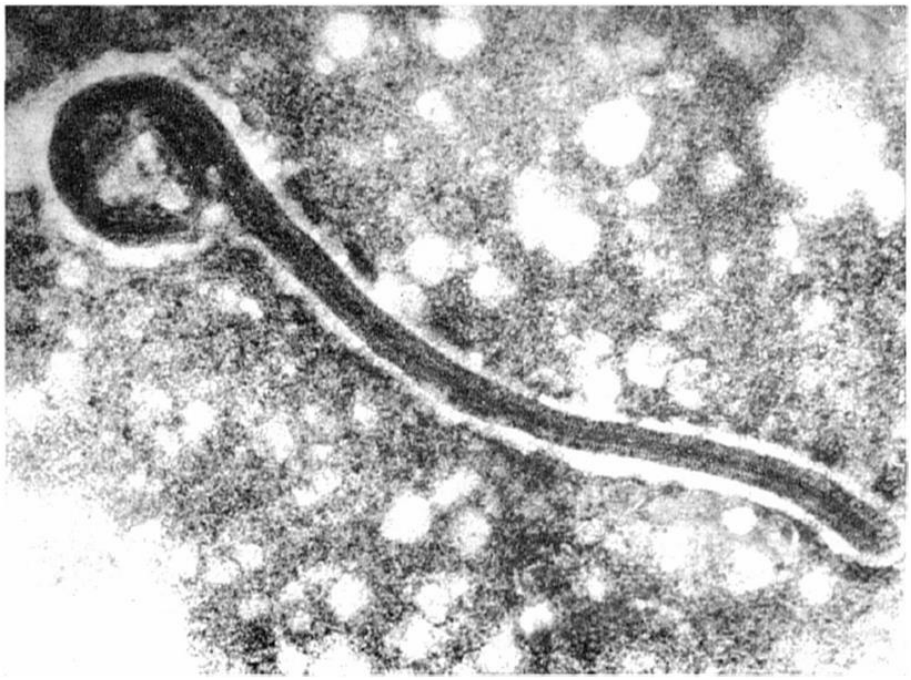

Figure 2-6: The first electron micrograph of a Marburg virion from 1967 (Brauburger *et al*., 2012).

Antibody detection methods have the disadvantage that antibodies are not present at detectable levels in early disease, or in patients with very severe disease. Furthermore, IgG antibodies are detectable for several years in patients who recover, and are therefore not always associated with active infection (Bannister, 2010). Detection of virus-specific antibodies is a common approach for determining the contact of a virus with an animal species; however, lack of specific reagents for the majority of wild animal species has limited its use (Feldmann *et al*., 2004).

Antigen detection is sensitive and can be used in early disease when viraemia is high and antibodies have not yet been produced (Ksiazek *et al*., 1999a).

2.4.1 Clinical signs

Patients normally present with non-specific signs, including fever, vomiting, diarrhoea, fatigue, myalgia, headache. Later on in the disease process, they may develop a rash. Some

affected individuals will show evidence of coagulation disorders with multiple foci of mucosal haemorrhage and persistent bleeding from injection sites, while massive haemorrhages usually only occur in fatal cases (Kortepeter *et al*., 2011; Baize *et al*., 2014).

2.4.2 ELISA

The most commonly used antibody detection method is ELISA for detection of IgG and IgM responses. Assays that have been developed include IgG and IgM capture ELISAs to detect EBOV, SUDV and RESTV antibodies (Ksiazek *et al*., 1999b); use of truncated rNPs to EBOV and RESTV for the detection of IgG responses to the respective viruses (Ikegami *et al*., 2003b); and filovirus species specific ELISAs for the detection of anti-GP IgG (Nakayama *et al*., 2010).

Antigen detection ELISA has been used to detect different filoviruses, using mAbs to NP for the detection of EBOV and RESTV (Niikura *et al*., 2001), RESTV (Ikegami *et al*., 2003a) and marburgvirus (Saijo *et al*., 2005; 2006a) and mAbs to VP40 to detect EBOV (Lucht *et al*., 2003). Antigen-capture ELISA methods are more sensitive and less prone to false-positive and false-negative results than other immunoassays (Bannister, 2010)

2.4.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

The RT-PCR is usually sensitive for conserved genes, but may fail to detect filoviruses if there is a considerable sequence variation (Bannister, 2010). This was the case in the first outbreak of BDBV, where initial tests using a highly sensitive real-time RT-PCR assay specific for EBOV, SUDV and marburgviruses were all negative (Towner *et al*., 2008).

The RT-PCR targets for filoviruses include the L, GP and NP genes (Dong *et al*., 2008). Various RT-PCR have been developed including a fluorogenic double-probe-based, one-step RT-PCR assay (Gibb *et al*., 2001a; Gibb *et al*., 2001b); real-time RT-PCR (Drosten *et al*.,

2002); Taqman RT-PCR (Weidmann *et al*., 2004); nested RT-PCR (Towner *et al*., 2004); multiplex PCR (Palacios *et al*., 2006); a consensus PCR (cPCR) method that utilizes a cocktail of specific primers in a one-step RT-PCR (Zhai *et al*., 2007); and a real-time RT-PCR for EBOV (Towner *et al*., 2007). Real-time RT-PCR has also been used to detect marburgvirus RNA extracted from formalin-fixed tissues (Bannister, 2010) and EBOV RNA from oral fluid specimen (Formenty *et al*., 2006). Multiplex RT-PCRs that have been developed include a prototype test kit real time RT-PCR for all filoviruses (Panning *et al*., 2007); a quantitative real-time quantitative Taqman™ RT-PCR (Trombley *et al*., 2010) and RT-PCR assay targeting the NP gene of filoviruses, that can detect all filovirus species (Ogawa *et al*., 2011).

2.4.4 Virus isolation

Virus culture is the gold standard as it provides for isolation of the virus for characterisation. However, due to the highly pathogenic nature of the viruses, virus culture can only be carried out in accredited BSL-4 containment facilities (Bannister, 2010). Ebolaviruses and marburgviruses grow well in a large variety of cell lines. Commonly used cell lines are Vero or Vero E6 cells. The shipment of infectious specimens for virus isolation needs favourable conditions, such as a cold chain during the shipment until arrival, which is often difficult in developing countries (Saijo *et al*., 2006b). Virus culture takes several days and is expensive, time consuming and hazardous (Wang *et al*., 2011). While genome sequencing has greatly reduced the need for virus isolation, it is still necessary for research (Bannister, 2010).

2.4.5 Electron microscopy

Electron microscopy as the method for visualization of virus particles (Wang *et al*., 2011), was used to detect filovirus virions in the first outbreak of MVD using thin sections of

inoculated cell cultures (Kissling *et al*., 1968). This method was improved for use in immunoelectron microscopy (Geisbert and Jahrling, 1990) and later refined to detect presence of filovirus in fluid specimens, reducing the length of the procedure (Geisbert *et al*., 1991). While this method has been termed 'rapid', multiple specimen cannot be examined quickly; the procedure is expensive, with highly complex equipment and specialised personnel required; and for visualisation, high concentrations of the viral particles are needed (Gibb *et al*., 2001a , Wang *et al*., 2011).

2.4.6 Reverse transcription-loop-mediated isothermal amplification (RT-LAMP)

The amplification of viral RNA using a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) method specific for EBOV (Kurosaki *et al*., 2007) and for marburgviruses (Kurosaki *et al*., 2010) was developed. This test is rapid and easy to use under field conditions, but is not as sensitive as RT-PCR (Kurosaki *et al*., 2010).

2.4.7 Other immunoassays

Indirect immunofluorescence antibody tests (IFAT) have also been used to detect filovirus infections. A polyvalent IFAT for detection of Lassa fever, EBOV and MARV was developed by Johnson *et al*. (1981). This involved preparation of live viruses to use for antigen detection, requiring BSL-4 containment facilities. This method was improved by using recombinant NP to Ebola virus to detect IgG response, thereby removing the need for a BSL-4 containment facility (Saijo *et al*., 2001b). IFAT for detection of IgM antibodies are sensitive, but high levels of antibodies to other diseases like malaria can affect the tests due to non-specific antigen binding (Bannister, 2010). It is also not possible to confirm IFAT results using other immunologic tests (Ksiazek *et al*., 1999b).

The radioimmunoprecipitation assay (RIPA) and the Western blot measure antibody responses to individual viral proteins in slightly different ways and offer the advantage of showing the molecular specificity of the response. Because the tests are too cumbersome to be used as primary serologic tools, they are most commonly used for confirmatory diagnosis and in research (Ksiazek *et al*., 1999b).

Several other immunoassays for antigen detection have been developed including an immunofiltration based assay to detect EBOV using mAbs to VP40 (Lucht *et al*., 2007); and monoclonal affinity reagent sandwich assays (MARSA) using Llamma single domain antibodies produced from mAbs to EBOV and marburgvirus NP (Sherwood *et al*., 2007; Sherwood and Hayhurst, 2013). Immunohistochemical staining of the skin for detection of EBOV particles has also been done (Zaki *et al*., 1999).

2.5 Treatment and control

Currently, there are no licenced vaccines or post exposure treatment available for filovirus infections (Geisbert and Feldmann, 2011; Mire *et al*., 2014). The key immunogenic protein in vaccine protection appears to be GP, with variable contribution from NP (Geisbert *et al*., 2010a).

2.5.1 Vaccination

Populations that require a preventive vaccine are those in endemic and outbreak areas; health care workers in outbreak areas; outbreak response teams; laboratory workers involved in filovirus research and defence and related personnel susceptible to the use of filoviruses as bio-weapons (Geisbert *et al*., 2010a). When a filovirus outbreak occurs, the actual virus causing disease is not immediately known; therefore the ideal vaccine would be the one that can protect against all known filoviruses lethal to humans (Swenson *et al*., 2008).

Vaccination experiments have been carried out in rodent models as well as NHPs with varying degrees of success. Disadvantages of using rodent models is that the pathogenesis and inflammatory responses in rodents are not exactly similar to those in primates, therefore data derived from these studies may not correlate with human disease (Geisbert *et al*., 2010a).

2.5.1.1 DNA vaccines

DNA vaccines expressing various MARV and EBOV viral proteins have been evaluated as potential vaccines. Guinea pigs immunized with plasmids encoding EBOV GP were challenged two and four months post immunisation and had 100 and 80 percent survival rates, respectively (Xu *et al*., 1998). Studies with guinea pigs immunized with plasmids encoding MARV GP showed 80 percent protection following challenge three months post immunisation (Hevey *et al*., 2001).

2.5.1.2 Virus-like particles

The VLP vaccine produced by transfection of 293T cells with plasmids expressing GP and VP40 has been shown to be efficacious in rodents and NHPs (Martins *et al*., 2013). The primary immunogenic component of the VLP is most likely GP (Licata *et al*., 2004). Mice vaccinated three times at three-week intervals with EBOV VLP were completely protected from a high dose challenge with EBOV (Warfield *et al*., 2003), while guinea pigs with a similar vaccination regimen with MARV VLP also elicited complete protection from MARV infection (Warfield *et al*., 2004). A pan-filovirus vaccine was then developed using VLPs to both EBOV and MARV which provided over 90 percent protection to guinea pigs challenged with either EBOV or MARV 28 days after a single intramuscular immunisation (Swenson *et al*., 2005). Consequently, NHPs intramuscularly vaccinated with the EBOV VLP three times, with intervals of 42 days, and then challenged four weeks later, were completely protected (Warfield *et al*., 2007). Recently, this EBOV VLP vaccine was evaluated in captive chimpanzees, and was found to be safe, not causing any symptoms of clinical disease with

the animals developing a robust immune response. Passive transference of the IgG from these chimpanzees to mice had a protective effect to mice challenged with EBOV (Warfield *et al*., 2014).

2.5.1.3 Viral vector approaches

A replication-competent recombinant vesicular stomatitis virus vaccine expressing MARV GP (rVSV-MARV-GP), EBOV GP (rVSV-EBOV-GP) or BDBV GP (rVSV-BDBV-GP), in which the VSV glycoprotein (G) was replaced by the respective filovirus GP was developed. A single intramuscular (i.m.) injection was shown to induce a strong humoral immune response in NHPs and gave complete protection to i.m challenge with homologous virus 28 days later (Jones *et al*., 2005; Mire *et al*., 2013). There was 100 percent survival rate in vaccinated NHPs exposed to homologous aerosol challenge 28 days post immunisation (Geisbert *et al*., 2008a). The rVSV-MARV-GP vaccine was able to confer cross protection to NHPs challenged with MARV-Angola and RAVV viruses, respectively (Daddario-DiCaprio *et al*., 2006a), and also conferred complete protection to NHPs given a homologous challenge 14 month post immunisation (Mire *et al*., 2014). It was also shown that rVSV-EBOV-GP could confer 75 percent cross protection against challenge with BDBV (Falzarano *et al*., 2011). As the rVSV is replication-competent, there is a concern on the safety of the vaccine (Feldmann and Geisbert, 2011).

Bivalent rabies virus (RABV)/EBOV vaccine was developed for protection against both rabies and EBOV. Vaccination of mice with replication competent, replication deficient or chemically inactivated RABV expressing EBOV GP conferred variable protection from EBOV challenge 77 days post immunisation, with the different vaccines giving 30 – 100 percent protection (Blaney *et al*., 2011). This was followed by immunisation and challenge of NHPs with the same viruses. Replication competent virus vaccine conferred 100 percent

protection to challenge, while the other vaccines gave 50 percent protection (Blaney *et al*., 2013).

Recombinant replication deficient adenovirus-serotype-5 expressing EBOV GP (rAd5-GP) and EBOV NP (rAd5-NP) was used to immunise NHPs, with a booster at nine weeks later, followed a week later by challenge with EBOV, with the vaccinated animals all surviving this challenge (Sullivan *et al*., 2003). Further experiments with this vector suggested that rAd5-NP was not necessary for development of immunity (Sullivan *et al*., 2006). The efficacy of rAd5 vector vaccines is reduced by pre-existing immunity to the vector. Another challenge is the high doses required (Feldmann and Geisbert, 2011). Adenovirus vectors with low seroprevalence in humans, rAd26 and rAd35, were used to make an EBOV-GP vaccine. The NHPs were inoculated with rAd26-GP, boosted a month later with rAd35-GP and challenged with EBOV four weeks later. All animals survived the challenge (Geisbert *et al*., 2011). A complex adenovirus vector, able to express multiple antigens was used to produce a multivalent-filovirus vaccine, against EBOV, MARV and SUDV. Two NHP groups challenged either with ebolaviruses or marburgviruses 15 weeks after booster immunisation were fully protected and also survived re-challenge with marburgviruses and ebolaviruses, respectively (Swenson *et al*., 2008).

Venezuelan equine encephalitis (VEE) replicons encoding EBOV NP were used to immunise mice, with one or two boosters, a month apart, followed by lethal challenge a month after last booster. It conferred protection in 75 – 80 percent of the mice. The NP induced a cytotoxic T-lymphocyte response, suggesting that for adequate protection by vaccines, it would be important to include proteins that induce such a response (Wilson and Hart, 2001).

Paramyxo viruses have also been used as vaccine vectors. Recombinant human parainfluenza type 3 virus (HPIV3) was modified to express either EBOV GP (HPIV3/GP) or EBOV GP

and NP (HPIV3/GP-NP). Guinea pigs intranasally immunized with these viruses were challenged 28 days later, with EBOV, and there was 100 percent survival (Bukreyev *et al*., 2006). Two dose intranasal immunisation of NHPs with HPIV3/GP, 28 days apart, conferred total protection of animals challenged with EBOV on day 67 (Bukreyev *et al*., 2007). One challenge to the use of these vaccine viruses are the levels of pre-existing immunity. Another concern is the safety of using a replication competent virus in humans (Feldmann and Geisbert, 2011).

2.5.1.4 Combined approaches

Combinations of DNA and viral vector vaccines have been attempted. The NHPs vaccinated with plasmids containing ebolavirus GP and NP cDNA (pGP, pNP), then boosted with recombinant replication deficient adenovirus expressing EBOV GP (rAd5-EBOV-GP) were challenged several weeks later with 100 percent survival (Sullivan *et al*., 2000). In a subsequent experiment, NHPs were vaccinated with GP expressing plasmid followed by a booster with rAd5 vectors containing the EBOV and SUDV GP genes. The NHPs immunised with this DNA prime/rAd5 EBOV/SUDV vaccine regime survived lethal challenge with BDBV, suggesting that there was cross protection (Hensley *et al*., 2010).

Guinea pigs immunized with plasmids expressing MARV GP on day 0, followed by booster immunisation with a recombinant baculovirus expressing MARV GP on days 28 and 56 survived lethal challenge on day 77 (Hevey *et al*., 2001). Follow up studies using EBOV GP instead of MARV GP did not yield positive results, with only 33 percent survival after homologous challenge (Mellquist-Riemenschneider *et al*., 2003).

2.5.2 Post-exposure treatment

Currently, the standard clinical treatment is supportive care with rehydration, nutritional support and symptomatic treatment (Jeffs *et al*., 2007; Roddy *et al*., 2011). Passive immuno

therapy has been suggested as a treatment option. There have been reports of successful treatment of patients with convalescent serum (Emond *et al*., 1977) or blood (Mupapa *et al*., 1999). It has been suggested that other factors than the presence of EBOV-specific antibodies in donor blood, contributed to this recovery (Mupapa *et al*., 1999; Sadek *et al*., 1999). No beneficial effect has been shown from the transfusion of convalescent-phase blood from NHPs immune to EBOV infection suggesting that whole blood transfusions are unwarranted (Jahrling *et al*., 2007).

The first complete post exposure protection of NHPs against a filovirus was achieved by administering rVSV-MARV-GP, 20-30 minutes after a high-dose MARV challenge (Daddario-DiCaprio *et al*., 2006b). Similar studies using rVSV-EBOV-GP as a post exposure treatment to EBOV challenge did not yield as good result, with all treated NHPs developing disease and a 50 percent survival rate (Feldmann *et al*., 2007). Another study using rVSV-SUDV-GP had better outcomes, with all treated NHPs surviving SUDV infection (Geisbert *et al*., 2008b). Subsequent studies administering rVSV-MARV-GP to NHPs either 24 hours or 48 hours after infection resulted in survival rates of 83 percent and 33 percent, respectively (Geisbert *et al*., 2010b). A scientist who pricked herself with a needle that had just been used to infect mice with EBOV was given post exposure treatment with rVSV-EBOV-GP with no adverse effects (Tuffs, 2009). Efficacy in this case is hard to prove as it is not known whether infection had actually occurred (Feldmann, 2010). In order for such a treatment to be effective, the filovirus species causing the disease has to be known, as there is little or no cross reactivity between different filoviruses. Another factor affecting the efficacy of this treatment is the time of commencement of treatment after exposure to the virus (Feldmann and Geisbert, 2011).

Some drugs have also been developed for treatment the of filovirus infections. A potent anticoagulant, recombinant nematode anticoagulant protein c2 (rNAPc2), was evaluated for treatment of EBOV infected NHPs, but showed limited efficacy, with a survival rate of 33 percent (Geisbert *et al*., 2003). Further studies with rNAPc2 showed even less efficacy against MARV infection (Geisbert *et al*., 2007). A pyrazine carboxamide derivative, T-705 (favipiravir), was administered to mice six days after infection with EBOV, and it resulted in complete treatment of all the infected mice (Oestereich *et al*., 2014). A synthetic adenosine-analogue, BCX4430 protected NHPs, 48 hours after infection with MARV (Warren *et al*., 2014).

Small interfering RNAs (siRNAs) inhibit viral replication. Guinea pigs treated with siRNAs targeting the EBOV polymerase (L) gene an hour after infection with EBOV were completely protected (Geisbert *et al*., 2006). NHPs given seven treatments of siRNAs targeting EBOV L, VP24 and VP 35 genes, 30 minutes after EBOV challenge and then daily for the next six days, survived, although they developed mild clinical signs of the disease (Geisbert *et al*., 2010c). This treatment is administered intravenously, which would not be practical in a normal outbreak in remote and resource poor areas. In order to achieve successful treatment, the particular filovirus species causing infection would have to be known (Feldmann and Geisbert, 2011).

Filoviral-specific mAbs have been used for post exposure experiments. NHPs were infected with a high dose of EBOV, and then treated with intravenous administration of a combination of three mAbs against EBOV GP, three doses, three days apart, starting either 24 hours or 48 hours post infection (p.i.). There was 100 percent protection in the group administered first, while the NHPs treated 48 hours p.i. had 50 percent survival rate. This was the first report of successful post exposure treatment administered more than an hour p.i. (Qiu *et al*., 2012a).

The surviving NHPs were then re-challenged with EBOV, ten weeks after the initial challenge and they all survived (Qiu *et al*., 2013a). To extend the post exposure window further, in addition to the EBOV GP mAbs treatment at three day intervals beginning from three days p.i., NHPs were treated with human IFN-α together with the first mAb treatment, and 75-100 percent of the NHPs survived, while 50 percent survived if given IFN-α treatment one day p.i. and mAbs treatment at four, seven and ten days p.i. (Qiu *et al*., 2013b).

Human-mouse chimeric mAbs to EBOV GP were administered intravenously to three NHPs 24 hours prior to and 24 and 72 hours after challenge with a lethal dose of EBOV. One NHP did not develop any disease while the two others succumbed to the disease, with one showing a delayed disease progression, suggesting that these mAbs provided partial protection against infection (Marzi *et al*., 2012).

Another group used a mAb cocktail of three mAbs to EBOV GP, MB-003, to treat NHPs challenged with EBOV, at one hour p.i. and two days p.i., with survival rates of 100 percent and 67 percent respectively (Olinger, Jr. *et al*., 2012). The MB-003 mAbs were then used therapeutically, after infected NHPs had a positive RT-PCR result for EBOV RNA and a fever. All the animals developed varying levels of the disease with 43 percent survival (Pettitt *et al*., 2013).

There are several challenges to the use of antibody therapy. Genetic variability of the virus may result in change of the peptide sequence of the antibody epitope, resulting in loss of efficacy of the vaccine. Another drawback is that antibody therapy can result in production of escape mutants, and may also cause antibody-dependent enhancement of the infection (Feldmann and Geisbert, 2011).

As outlined above, there have been several promising studies on various therapies. Looking at the rapid progression of disease, a combination of various therapies would most likely be more effective, with treatments to reduce viral replication there by delaying fatalities, in order to provide sufficient time for a potential post exposure treatment to be effective (Feldmann and Geisbert, 2011; Kondratowicz and Maury, 2012).

2.6 Filoviral monoclonal antibody studies

Production of mAbs by fusion of mice myeloma and mice spleen cells to form immortal cell lines of hybridoma cells was first described in 1975 (Kohler and Milstein, 1975). Briefly, mice were immunized with a specific antigen resulting in spleen cells producing antibody reactive to the immunizing antigen. These cells were then fused with myeloma cells and grown in hypoxanthine-aminopterin-thymidine (HAT) medium, resulting in hybridoma cells secreting antibodies. Myeloma cells are hypoxanthine-guanine phosphoribosyl transferase (HGPRT)-negative, while spleen B cells are HGPRT-positive. Only HGPRT-positive cells can grow in HAT medium, so unfused myeloma cells die. Unfused spleen cells also die because they cannot replicate, leaving only hybridoma cells in the culture (Alkan, 2004). Specific hybridoma cells are then selected and cultured, resulting in a cell line secreting the same antibody.

Several different studies have produced mAbs against various filovirus proteins for use in experimental studies, diagnostics and treatment. Some mAbs have been developed to investigate antibody-dependent enhancement of EBOV, RESTV (Takada *et al*., 2007a) and MARV infection (Nakayama *et al*., 2011). Ebola virus GP- and VP40-specific mAbs (Shahhosseini *et al*., 2007) and EBOV NP-specific mAbs (Wang *et al*., 2012) that can be used potentially for diagnostics or therapy have also been developed. The mAbs developed for diagnostic purposes include EBOV NP-specific mAbs (Niikura *et al*., 2001; Niikura *et al*.,

2003), EBOV VP40-specific mAbs (Lucht *et al*., 2003) and EBOV GP-specific mAbs (Lucht *et al*., 2004), MARV NP-specific mAbs (Saijo *et al*., 2005) and SUDV GP-specific mAbs (Yu *et al*., 2006). Ebola virus GP-specific mAbs have also been developed and used in various pre- and post-exposure treatment studies in both rodent and NHP models (Wilson *et al*., 2000; Takada *et al*., 2007b; Qiu *et al*., 2011; Marzi *et al*., 2012; Olinger, Jr. *et al*., 2012; Qiu *et al*., 2012a; Qiu *et al*., 2012b; Qiu *et al*., 2013b).

There is a relatively long turnaround time of the tests developed using the mAbs described in the above studies. Because of this, there is need for production and application of specific mAbs for use in rapid diagnosis of filovirus infections.

2.7 Immunochromatographic strip tests

Immunochromatographic strip tests are lateral flow immunoassays (LFIA) used to detect the presence of a target (antigen or antibody) using specifically labelled capture molecules (antigen or antibody) (Holford *et al*., 2012; Karakus and Salih, 2013). There are two types of immunochromatographic strip tests, sandwich and competitive (inhibition) formats. The sandwich format is used to detect target analytes with multiple epitopes, while the competitive format is used when the target has only one epitope (Posthuma-Trumpie *et al*., 2009; Hu *et al*., 2014).

Advantages of immunochromatographic strip tests include the ease of use, rapid time to results, low cost relative to other standard tests with little or no equipment or technical training required, portability, and long shelf life at room temperature (Linares *et al*., 2012; Karakus and Salih, 2013; Leonardi *et al*., 2013). Other advantages are that they can be used as point of care diagnostics, they can be produced in large batches with good reproducibility, and do not get contaminated with previously tested sample (Posthuma-Trumpie *et al*., 2009).

Immunochromatographic strip tests have several disadvantages. The sensitivity of these tests is normally lower compared to other conventional techniques like PCR (Sithigorngul *et al*., 2011; Hu *et al*., 2014). They do not give quantitative results; there is decreased precision and limited sensitivity due to imprecise sample volumes; it can usually only test for one analyte, membrane pores can get blocked, and pre-treatment of non-fluid sample is necessary (Posthuma-Trumpie *et al*., 2009). Interpretation of the results is subjective and depends on the strength of the colour signal, which when faint can easily be misinterpreted as negative (Leonardi *et al*., 2013; Moore, 2013)

Several immunochromatographic strip tests for the detection of antigen using mAbs have been produced and validated for field tests for other viral diseases. These include rinderpest virus (Bruning *et al*., 1999; Wambura *et al*., 2000; Bruning-Richardson *et al*., 2011); foot and mouth disease virus (FMD) (Reid *et al*., 2001; Ferris *et al*., 2009; 2010a); dengue virus (Edwards and Baeumner, 2006); porcine rota virus (Kang *et al*., 2007); avian influenza (Peng *et al*., 2008); H5 subtype of highly pathogenic avian influenza (Cui and Tong, 2008); Japanese encephalitis virus (Li *et al*., 2010); swine vesicular disease virus (Ferris *et al*., 2010b) and porcine reproductive and respiratory syndrome virus (Cui *et al*., 2008; Li *et al*., 2011). Unfortunately, however, such similar test has been produced for FHF diagnosis to date.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design

The aim of the study was to produce mAbs to ebolavirus NP, which could then be used in an immunochromatographic strip test for diagnosis of ebolavirus infections under field conditions.

The filoviruses used in this study were: EBOV (strain Mayinga), SUDV (strain Boniface), TAFV (strain Côte d'Ivoire), BDBV (strain Bundibugyo), RESTV (strain Pennsylvania), MARV (strains Angola, Musoke, Ozolin and Ci67), RAVN and LLOV. These were the virus strains available in the laboratory. VLPs and recombinant proteins were produced from plasmids expressing filoviral genes. Mice were immunized with EBOV VLPs. Spleen cells from one immunized mouse were then fused with myeloma cells to produce hybridomas expressing antibodies. Specific hybridomas expressing anti-EBOV NP antibodies were selected and cloned to produce hybridoma colonies expressing anti-EBOV NP mAbs. Selected hybridomas were produced in larger quantities using mice-ascites fluid technique, and the resultant mAbs purified and characterised. A selected mAb was then used to produce an immunochromatographic strip test. This test was evaluated with both VLPs and authentic virus lysates. All procedures were carried out in BSL-2 and BSL-4 containment laboratories where appropriate. Standard protocols were followed for all assays, with some modifications where necessary. All reagents used were of laboratory analytical grade. Animal studies were carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The protocol was approved by the Hokkaido University Animal Care and Use Committee. All efforts were made to minimize the suffering

of animals. All infectious work with filoviruses was performed under high containment complying with standard operating procedures approved by the Institutional Biosafety Committee in the BSL-4 Laboratories of the Integrated Research Facility at the Rocky Mountain Laboratories, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA.

3.2 Plasmid construction

Plasmids expressing filoviral GP, VP40 and NP were constructed as described previously (Nakayama *et al*., 2010; Nidom *et al*., 2012). Briefly, viral RNAs were extracted from the supernatant of Vero E6 cells infected with EBOV, SUDV, TAFV, BDBV, RESTV or MARV (strain Angola). Full length NP, VP40 and GP cDNAs were amplified by RT-PCR using KOD-plus-Neo polymerase (Toyobo) and run on 0.8 percent agarose gel. The resulting PCR product was purified using Wizard® SV Gel & PCR Clean-up System (Promega) and cloned into TOPO® cloning vector using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen). Plasmid vectors were transformed into *Escherichia coli* TOP 10 competent cells (Invitrogen), grown on LB agar with Kanamycin (Sigma-Aldrich) and incubated at 37°C while shaking for 20 hours. Colonies were selected and cultured in tubes containing LB broth with Kanamycin and incubated at 37°C while shaking for 20 hours. The cDNA was purified from the culture using Wizard® Plus Minipreps DNA Purification System (Promega). After sequence confirmation, the cloned genes were inserted into the mammalian expression vector pCAGGS. The pCAGGS vector and TOPO® vectors containing cDNA insert were digested with the relevant restriction enzymes, run on 0.8 percent agar gel and purified with Wizard® SV Gel & PCR Clean-up System (Promega). The cDNA-fragment and pCAGGS were mixed at a ratio of 10:1, together with ligase and salt solution and transformed into *E. coli* TOP 10 competent cells. Transformed cells were plated on LB agar with penicillin (Gibco, Life

Technologies) and incubated at 37°C overnight. Colonies were selected and cultured in tubes containing LB broth with penicillin and incubated at 37°C while shaking for 18 hours. Plasmids with cDNA insert were purified from the culture using Wizard® Plus Minipreps DNA Purification System (Promega). Plasmid sequencing was done to confirm the homology of the ebolavirus gene insert.

3.3 Sequencing of cloned filoviral genes

The target gene insert in both TOPO® and pCAGGS plasmids was processed from the plasmids using restriction enzymes and purified using Wizard® SV Gel & PCR Clean-up System (Promega). For sequencing, BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) was used. Briefly, cycle sequencing of the gene involved initial activation at 96°C for one minute, followed by 25 cycles of denaturation at 96°C for ten seconds, annealing at 50°C for five seconds and extension at 60°C for four minutes. After Ethanol/EDTA/Sodium acetate precipitation, the sample was dissolved in Hi-Di™ Formamide (Applied Biosystems) and capillary electrophoresis conducted using 3130xL Genetic Analyser (Applied Biosystems). The raw data was analysed with ATGC software, and compared to template sequence using Genetyx ver.10 (Genetyx Corporation).

3.4 Preparation of purified VLPs and NP

Human embryonic kidney 293T cells were grown in Dulbeco's modified Eagle's medium (DMEM, Sigma-Aldrich), supplemented with ten percent foetal calf serum (FCS) (CCB, Nichirei Bioscience), penicillin (100 unit/ml) and streptomycin (100 µg/ml) (Gibco, Life Technologies). The VLPs for each filovirus used in this study were produced by the transfection of 293T cells with plasmids expressing NP and VP40 together with or without the plasmid expressing GP as described previously (Licata *et al*., 2004; Urata *et al*., 2007). Forty-eight hours after transfection, VLPs in the supernatant were purified by centrifugation

through a 25 percent sucrose cushion at 28,000 x *g* and 4°C for 1.5 hours. The pelleted VLPs were re-suspended in phosphate-buffered-saline (PBS) and stored at -80°C. The presence of recombinant proteins was confirmed by SDS-PAGE analysis.

For the preparation of purified recombinant NP, 293T cells transfected with the plasmid encoding ebolavirus NP were lysed by adding lysis buffer (10 mM Tris·HCl (pH 7.8), 0.15 M NaCl, 1 mM EDTA, 0.1 percent Nonidet P-40 and Protease inhibitor mixture (Roche)) and incubating at room temperature for an hour. The NP fraction was collected by discontinuous 25-40 percent (wt/wt) CsCl gradient centrifugation of the lysates at 200,000 x *g* at 4°C for one hour as described previously (Noda *et al*., 2010; Bharat *et al*., 2012). The supernatant was collected in 0.5 ml aliquots, and the protein content was measured. Aliquots with low to medium protein concentration were combined and centrifuged at 200,000 x *g*, at 20°C for one hour. The supernatant was discarded and the pellet was resuspended with PBS containing protease inhibitor and subjected to SDS-PAGE analysis to confirm the presence of NP.

3.5 Mouse mAb production

3.5.1 Mouse immunisation

On day zero, six-week-old female Balb/c mice were immunized intramuscularly with 100 µg of EBOV VLPs consisting of NP and VP40 with complete Freund's adjuvant (Difco). The animals were boosted intramuscularly on day 14 with 100 µg of EBOV VLPs and incomplete Freund's adjuvant. Antibody titres to EBOV VLP on day 25 were determined using indirect ELISA with serial dilutions of mouse sera. The mouse with the highest antibody titre was given a final intravenous boost with 100 µg of EBOV VLPs without adjuvant on day 39.

3.5.2 Hybridoma production

Hybridomas were produced according to the standard procedure (Shahhosseini *et al*., 2007). Spleen cells were harvested on day 42 and fused to P3-U1 myeloma cells using polyethylene glycol (PEG), followed by dilution with Roswell Park Memorial Institute medium (RPMI) 1640 (Gibco, Life Technologies) containing ten percent hypoxanthine-aminopterin-thymidine (HAT) (Gibco, Life Technologies), 20 percent FCS, penicillin (100 unit/ml), streptomycin (100 µg/ml), L-glutamine (4 mM) (Gibco, Life Technologies) and 2-mercaptoethanol (2ME) (55 µM) (Gibco, Life Technologies). Feeder spleen cells from naïve mice were added and the cell mixture was plated into 96-well plates and incubated at 37°C and five percent CO_2. The medium was changed every two days, for ten days, with HAT concentration reduced to 0 percent in the final medium change.

On day 11, hybridoma supernatants were screened by ELISA for the secretions of EBOV NP (ZNP)-specific antibodies using purified EBOV NP antigens. Hybridomas from ZNP-antibody positive wells were further passaged in 24-well plates and maintained in RPMI 1640 containing 20 percent FCS, penicillin (100 unit/ml), streptomycin (100 µg/ml), L-glutamine (4 mM) and 2ME (55 µM).

Selected hybridoma cells were then cloned by limiting dilution in RPMI 1640 medium containing ten percent supplement (BM Condimed h1, Roche), 20 percent FCS, penicillin (100 unit/ml), streptomycin (100 µg/ml), L-glutamine (4 mM) and 2ME (55 µM) in order to obtain hybridomas producing single monoclonal antibodies (mAbs). The resulting cell clones were passaged in RPMI 1640 (Sigma-Aldrich) containing 20 percent FCS, penicillin (100 unit/ml), streptomycin (100 µg/ml), L-glutamine (4 mM) and 2ME (55 µM). Hybridoma supernatant was further screened by ELISA using VLPs of all known filoviruses to detect

cross-reactivity. Specificity and cross-reactivity of mAbs were also confirmed by Western blotting.

3.5.3 Purification of monoclonal antibodies

To produce ascites-derived mAbs, the selected hybridomas were inoculated intraperitonealy into Balb/c mice. Ascites was collected between day eight and 11 post inoculation. Each mAb was purified from ascites using Affi-Gel® Protein A MAPS® II Kit (BIO-Rad). Buffer exchange and antibody concentration was carried out on the purified samples using Amicon® Ultra-15 centrifugal filter device (Millipore) with a molecular weight cut-off of 10 kDa. ELISA was carried out to confirm the presence of mAbs to ZNP, while SDS-PAGE was used to confirm the purity of the mAbs. Isotyping of purified mAbs was done using Serotec Mouse mAb Isotyping Test Kit (AbD Serotec), following the manufacturer's instructions. Briefly, one µl of each mAb was added to 50 µl of one percent w/v bovine serum albumin and placed in development tubes containing antibody coated micro-particles. The isotyping strip was then placed in the development tube and the results read after ten minutes.

3.6 Production of rabbit antisera

Genetyx ver6.0 for Windows (GENETYX CORPORATION) was used to predict B-cell epitopes in the NPs of EBOV, SUDV, TAFV, BDBV, RESTV and MARV, and the aa positions around 630-650 were selected. Synthetic peptides corresponding to this aa region in NP were produced (Sigma-Aldrich). Rabbits were then immunized with keyhole limpet hemocyanin-conjugated synthetic peptides by the standard procedure, and polyclonal antisera were obtained on day 49.

3.7 Infection of Vero E6 cells with live filoviruses

Vero E6 cells cultured in DMEM supplemented with 10 percent FCS, penicillin (100 unit/ml), streptomycin (100 µg/ml) and L-glutamine (4 mM) were infected with EBOV (strain Mayinga), SUDV (strain Boniface), TAFV (strain Cote d'Ivoire), BDBV (strain Bundibugyo), RESTV (strain Pennsylvania), MARV (strains Angola, Musoke, Ozolin and Ci67) or RAVV (strain Ravn) at a multiplicity of infection of 1 and maintained for 72 hours. Western blot analysis was carried out on the supernatant and cell lysate to confirm specificity and cross reactivity of the mAbs and rabbit antisera.

3.8 Enzyme-linked immunosorbent assay

Each well on 96-well ELISA plates (Nunc®, Maxisorp) was coated with purified ZNP (2µg/ml), VLPs (2-5µg/ml) or synthetic peptides (100µg/ml) – in PBS (50 µl) overnight at 4°C. ELISA was carried out as described previously (Nakayama *et al*., 2011). Briefly, the coated plates were washed with PBS containing 0.05% Tween 20 (PBST). Three percent skim milk in PBST was added as a blocking buffer, and incubated at room temperature for two hours, followed by washing with PBST. Primary antibody was then added (mouse antisera, hybridoma supernatants, purified mAbs or rabbit antisera) and incubated at room temperature for one hour, followed by washing with PBST. Secondary antibodies (goat anti-mouse IgG (H+L) or donkey anti-rabbit IgG (H+L) conjugated with peroxidase (Jackson ImmunoResearch)) were then added and incubated at room temperature for one hour, followed by washing with PBST. Bound antibodies were visualized by adding 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich). The reaction was stopped by the addition of 1 N sulphuric acid to the mixture, and the optical density (OD) measured at 450 nm using an ELISA plate reader.

3.9 Sodium dodecyl sulphate - polyacrylamide gel electrophoresis

Samples were denatured by mixing equal volumes of SDS-PAGE sample buffer with 2ME (55 µM) and incubated at 95°C for four minutes and separated by electrophoresis on 10 percent SDS-PAGE.

To confirm the presence of recombinant proteins, VLPs, were subjected to SDS-PAGE and stained with 2D Silver Stain II 'DAIICHI' (Daiichi Pure Chemicals). To confirm the purity of EBOV NP in resuspended pellets, the samples were subjected to SDS-PAGE and stained with Quick-CBB PLUS (Wako Chemicals).

3.10 Western blot analysis

Vero E6 cells were infected with ebolaviruses or marburgviruses and lysates and supernatants were subjected to SDS-PAGE.

For the screening of hybridoma supernatants (see above), VLPs were used instead of authentic virus lysates. After electrophoresis, separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore) or Immobilon-P transfer membrane (Millipore). Mouse mAbs and rabbit antisera were used as primary antibodies. The bound antibodies were detected with peroxidase-conjugated goat anti-mouse IgG (H+L) or donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch), followed by visualisation with Immobilon Western (Millipore).

3.11 Epitope mapping

Synthetic peptides with seventy-three overlapping aa sequences, 20 aa in length, covering the entire aa sequence of ZNP (PEPscreen®, Sigma-Aldrich), were coated on ELISA plates at the

concentration of 100 µg/ml. Purified mAbs were used as primary antibodies, at the concentration of 1 µg/ml, and ELISA was carried out as described above.

3.12 Development of the immunochromatographic strip test

The immunochromatographic strip test was produced in collaboration with Denka Seiken Co. Ltd, Japan, using the previously described sandwich format (Bruning *et al*., 1999; Reid *et al*., 2001; Posthuma-Trumpie *et al*., 2009; Bruning-Richardson *et al*., 2011; Hu *et al*., 2014).

3.12.1 Selection of suitable mAbs

After screening for specificity, three ZNP mAbs were selected. In addition two other anti-GP mAbs were selected as negative controls, MGP14-22 (anti-MARV GP) and ZGP42/3.7 (anti-EBOV GP). These mAbs were then coated onto ELISA plates. These five mAbs were each conjugated to latex particles and then mixed with VLPs to EBOV, SUDV, TAFV, BDBV and RESTV. Each of the mAb and VLP mixtures was then applied to coated ELISA plates and examined for blue colour change. The ZNP mAb with the best overall reactivity was selected for use in the test.

3.12.2 Preparation of the immunochromatographic strip test device

The immunochromatographic strip includes four components: a sample pad, a conjugate pad, a nitrocellulose membrane and an absorbent pad. The sample pad was composed of cellulose filters and embedded with latex conjugated ZNP mAbs. The conjugate pad was composed of glass fibres. A nitrocellulose membrane was used for visualisation of the test. For the test line, ZNP31-1-8 mAbs was applied to the nitrocellulose membrane. To make the control line, rabbit-anti-mouse antibodies were applied, parallel to the test line. The absorbent pad was made from cellulose filters and functions to wick the fluid through the membrane to maintain

a continuous flow of the liquid (Fig. 3-1). This was mounted onto an adhesive card and placed in a plastic housing.

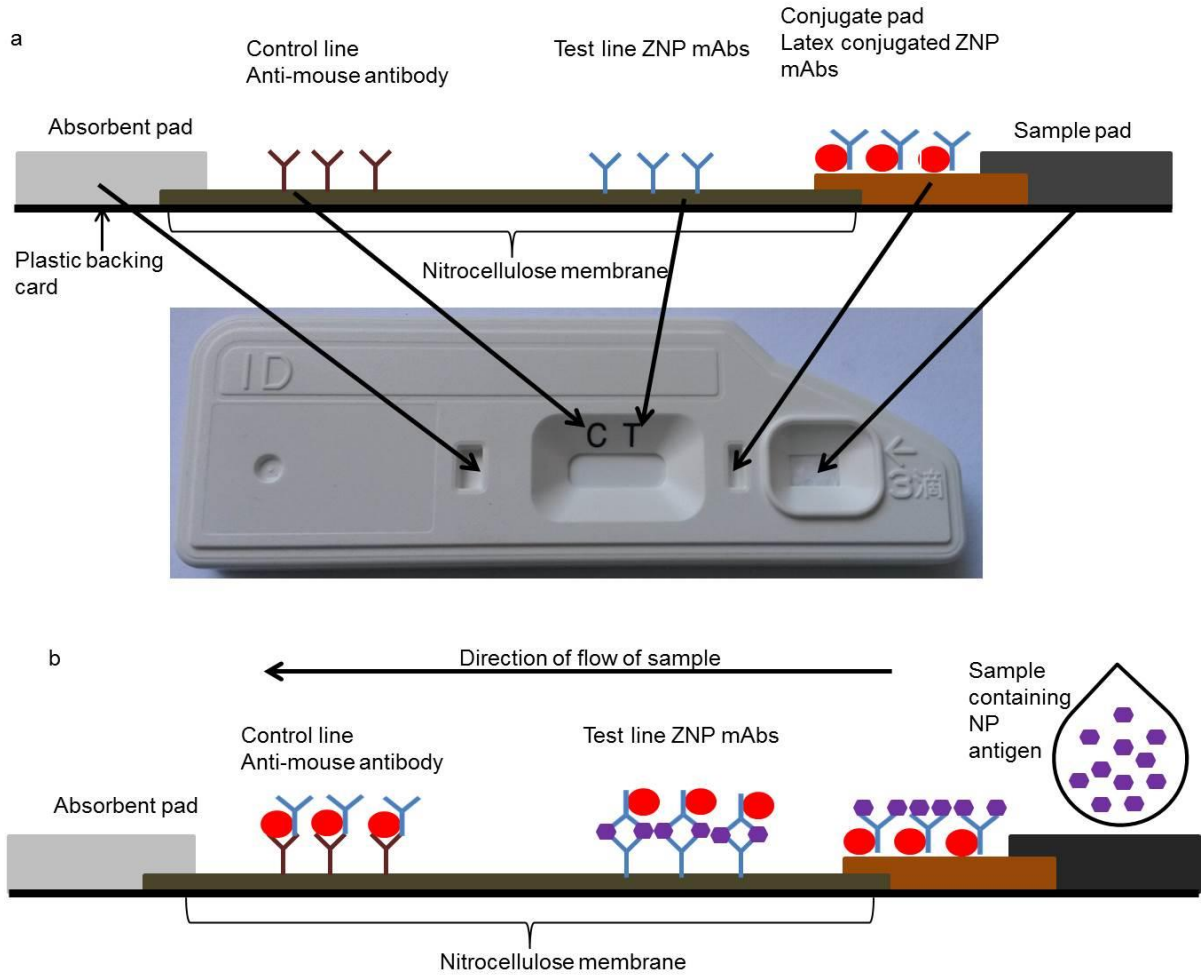

Figure 3-1: Representation of the immunochromatographic strip test showing the constituents of the strip before (a) and after (b) the addition of the sample containing the target antigen.

3.12.3 The principle and procedure of the immunochromatographic strip test

In test samples containing VLPs or authentic viruses, the antigen would bind to latex conjugated ZNP mAb and the resulting complex would be captured by the ZNP mAb at the test line (T) to give a blue dot. Any unbound mAb conjugated with latex moves across the test line to be captured by the rabbit anti-mouse antibody to form a blue band at the control line (C). In a sample with ebolavirus antigen, both the test line and the control line will be

visualized. In the sample without ebolavirus antigen or below the limit of detection for the test, only the control line is demonstrated. Thus, the appearance of colour change within 20 minutes; a blue spot in the test region and a blue line in the control region; indicates a positive result; whereas the appearance of only a blue line, in the control region, indicates a negative result (Fig. 3-2).

3.12.4 Testing the immunochromatographic strip test device with VLPs and authentic virus lysates

To test the device, VLPs consisting of the ebolavirus proteins were prepared for all known *Ebolavirus* species (i.e. EBOV, SUDV, TAFV, BDBV and RESTV) and used as surrogates of infectious virus particles. For negative control, MARV, LLOV and PCAGGS plasmids were used. A drop of a 1:1 dilution of sample buffer and VLP was placed on the sample pad and the results read after 20 minutes (Fig. 3-2).

To test with authentic viruses, supernatants containing infectious EBOV ($1X10^6$ ffu/ml), SUDV ($3X10^5$ ffu/ml), TAFV ($1X10^7$ $TCID_{50}$), BDBV ($1X10^5$ ffu/ml), RESTV ($7X10^5$ ffu/ml) or MARV ($1X10^5$ ffu/ml) prepared from infected Vero E6 cells were used.

Figure 3-2: Immunochromatographic strip test positive (a) and negative (b) windows

CHAPTER FOUR

4.0 RESULTS

4.1 The VLP inoculated mice

Of the three mice immunised with VLPs, serum IgG antibodies of Mouse #1 (M1) gave the highest OD value on day 25 and Mouse #2 (M2) gave the lowest value (Fig. 4-1). M1 was selected for hybridoma formation.

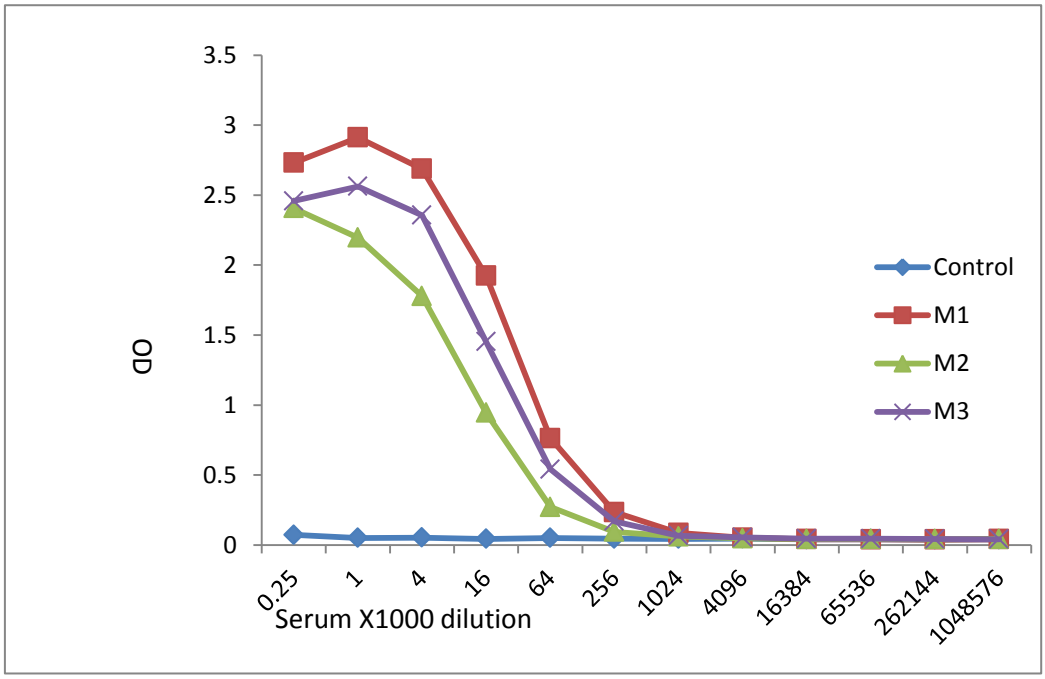

Figure 4-1: Anti-EBOV VLP IgG antibodies on day 25 detected by ELISA. ELISA plates were coated with the EBOV VLP antigen at a concentration of 10 µg/ml. Serial dilutions of mouse sera were used as primary antibody. Control serum was obtained from a naïve Balb/c mouse. Goat anti-mouse IgG (H+L) was used as secondary antibody.

4.2. Specificity and cross-reactivity of NP-specific mAbs

In the first screening process, 127 hybridomas producing antibodies reactive to the EBOV NP were obtained. None of them showed cross-reactivity to MARV NP. These antibodies were

further assessed by ELISA for their cross-reactivity with the recombinant NPs of the other known viruses in the genus *Ebolavirus* (SUDV, TAFV, BDBV and RESTV). Several different profiles for the cross-reactivities of these antibodies were found (Appendix A). Representative clones for each obtained cross-reactivity profile showing the highest OD_{450} values were selected and further cloned by limiting dilution.

There were some specificity changes during the cloning process. The mAbs ZNP24 and ZNP106 were negative for reactivity to SUDV, but positive for other ebolaviruses. After cloning, ZNP24-4-2 and ZNP106-9 additionally became weakly positive for SUDV. The mAb ZNP108 was not reactive to BDBV while its clone, ZNP108-2-5 detected it. Clone ZNP105-7 was able to detect TAFV when ZNP105 had tested negative for TAFV reactivity.

Ten clones of NP-specific mAbs were then established (ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2, ZNP106-9, ZNP108-2-5, ZNP105-7, ZNP98-7, ZNP35-16-3-5 and ZNP62-7). These clones were inoculated into mice, and the resulting mAb rich ascites collected and purified. All the mAbs were confirmed to be pure on SDS-PAGE analysis (Fig. 4-2).

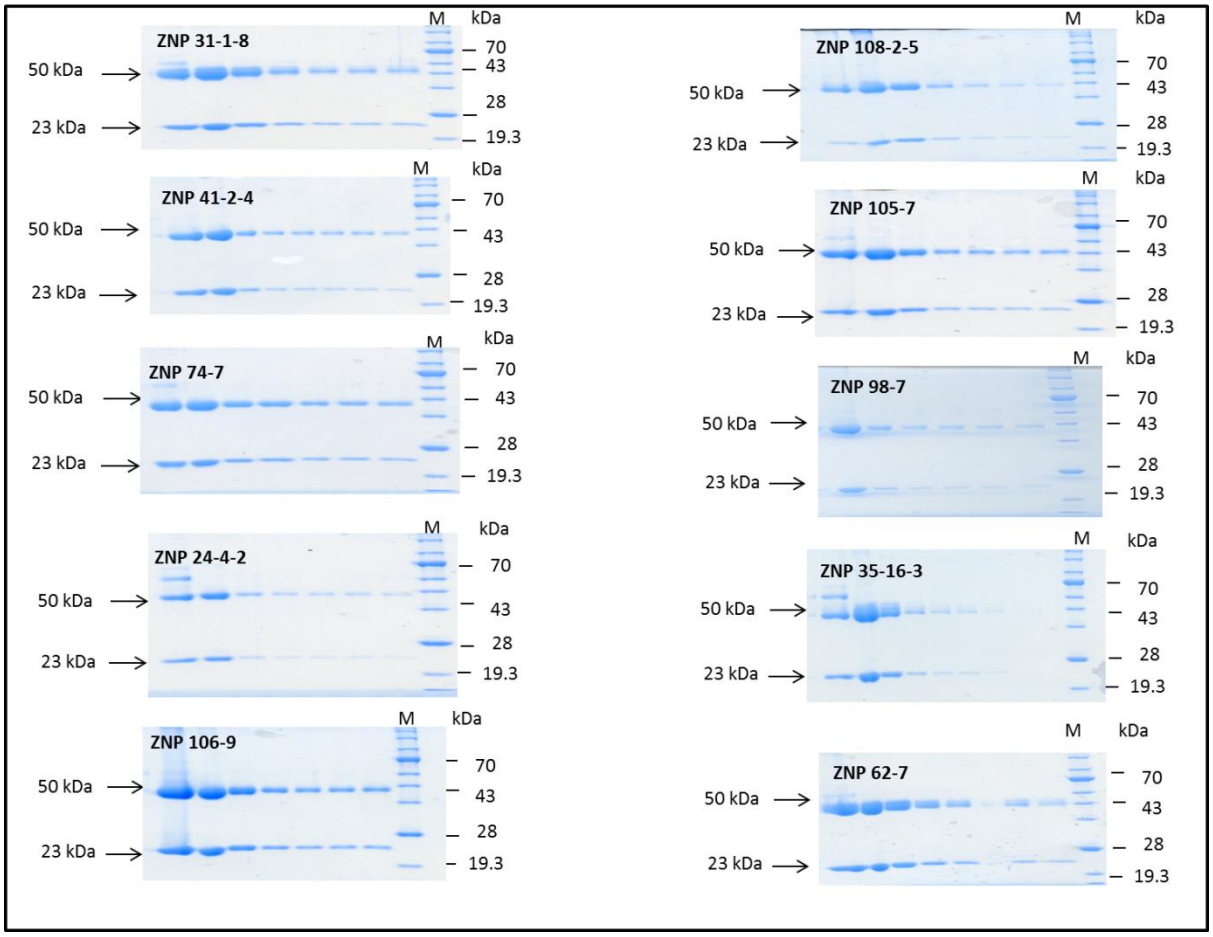

Figure 4-2: SDS-PAGE of purified mAbs. To confirm purity of mAbs (IgG) purified from ascites fluid, the eluates were subjected to SDS-PAGE and stained with Quick-CBB PLUS (Wako Chemicals). There was visualisation of two bands, of 50 kDa and 23 kDa, representing heavy and light IgG chains respectively.

These clones were divided into seven groups based on their cross-reactivity profiles in ELISA (Table 4-1). Four mAbs (ZNP31-1-8, ZNP41-2-4, ZNP74-7 and ZNP24-4-2) reacted with all known viruses representing each *Ebolavirus* species, with one (ZNP24-4-2) having relatively weak reactivity with SUDV. Four mAbs (ZNP106-9, ZNP108-2-5, ZNP105-7 and ZNP98-7) bound to NPs of some viruses in addition to EBOV, and two mAbs (ZNP35-16-3-5 and ZNP62-7) reacted only to EBOV.

Isotyping of the above mentioned clones revealed they were all IgG1 (Table 4-1), apart from ZNP98-7 (IgG2a) and ZNP62-7 (IgG2b).

Table 4-1: Cross reactivity profiles of mAbs

mAb (group)	Isotype	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
ZNP31-1-8 (I)	IgG_1	++*	++	++	++	++	-
ZNP41-2-4 (I)	IgG_1	++	++	++	++	++	-
ZNP74-7 (I)	IgG_1	++	++	++	++	++	-
ZNP24-4-2 (II)	IgG_1	++	+	++	++	++	-
ZNP106-9 (III)	IgG_1	++	+	++	++	-	-
ZNP108-2-5 (IV)	IgG_1	++	-	++	++	-	-
ZNP105-7 (V)	IgG_1	++	-	-	++	++	-
ZNP98-7 (VI)	IgG_{2a}	++	-	-	++	-	-
ZNP35-16-3-5 (VII)	IgG_1	++	-	-	-	-	-
ZNP62-7 (VII)	IgG_{2b}	++	-	-	-	-	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: $OD \geq 1.0$; +: $0.3 < OD < 1.0$; -: $OD \leq 0.3$.

Importantly, these different reactivity profiles enabled the distinguishing of all the known viruses belonging to different *Ebolavirus* species by using combinations of these mAbs: EBOV was recognised by all the mAbs, SUDV by ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2 and ZNP106-9; TAFV by ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2, ZNP106-9 and ZNP108-2-5; BDBV by ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2, ZNP106-9, ZNP108-2-5, ZNP105-7 and ZNP98-7; and RESTV by ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2 and ZNP105-7.

The reactivities of these NP-specific mAbs were further tested by Western blotting analysis using lysates of actual filovirus particles grown in Vero E6 cells (Fig. 4-3). It was found that the mAbs predominantly bound to proteins of approximately 100 kDa and some smaller

proteins. The cross-reactivity profiles and virus specificities were similar to those obtained by ELISA.

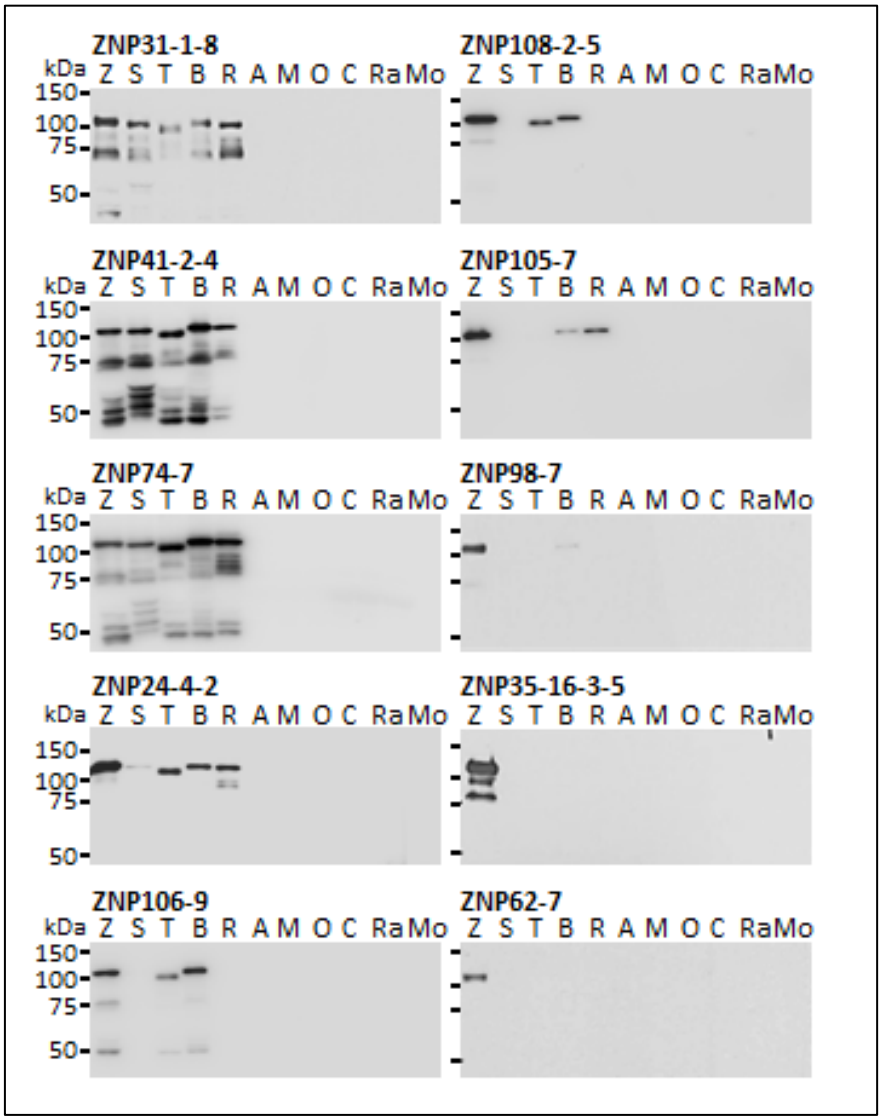

Figure 4-3: Reactivity of mouse mAbs in Western blot analysis. Vero E6 cells were infected with EBOV (Z), SUDV (S), TAFV (T), BDBV (B), RESTV (R), MARV Angola (A), MARV Musoke (M), MARV Ozolin (O), MARV Ci67 (C) or RAVV (Ra). Cell culture supernatants containing virus particles were collected, inactivated and subjected to SDS-PAGE under reducing conditions. Mo, mock-infected.

4.3 Synthetic peptide-based scanning to determine linear epitopes recognized by mAbs

To determine the epitopes recognised by the mAbs, their reactivities to synthetic peptides (20 aa in length) were analysed by ELISA (Fig. 4-4). The antigen peptides corresponded to 73 overlapping peptide sequences (ten aa overlapped between consecutive peptides) derived from EBOV NP and covered the entire aa sequence of this protein. This synthetic peptide-based scanning allowed the determination of some linear antigenic peptide sequences on EBOV NP.

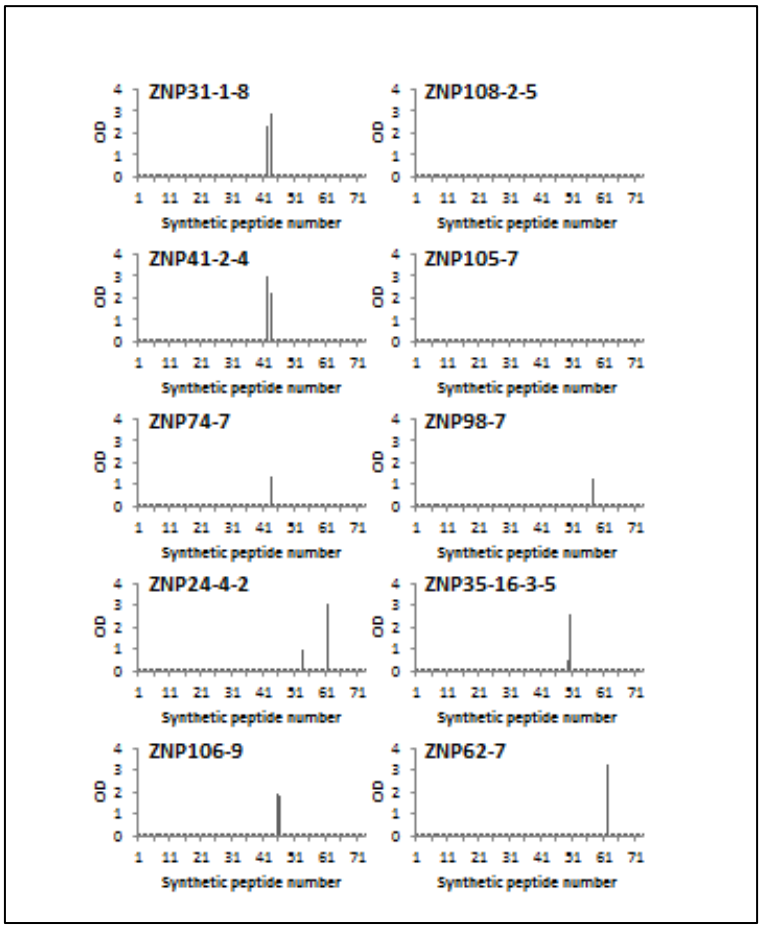

Figure 4-4: Reactivities of mAbs to EBOV NP-derived synthetic peptides. Seventy-three overlapping peptide sequences (20 aa in length with a ten aa overlap) covering the entire aa sequence of NP of EBOV Mayinga were coated on ELISA plates at a concentration of 100 µg/ml. Purified mAbs were used as primary antibodies at a concentration of 1µg/ml. OD measurements were determined at 450nm.

Of the ten mAbs described above, eight (ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2, ZNP106-9, ZNP98-7, ZNP35-16-3-5 and ZNP62-7) bound to at least one peptide, whereas two (ZNP108-2-5 and ZNP105-7) had no positive reaction. The aa sequences recognised by these eight mAbs are summarized in table 4-2 and figure 4-5.

Table 4-2: Amino acid sequences important for epitope formation.

mAb	Peptide sequences recognised by mAb	Amino acid positions
ZNP31-1-8	YDDDDDIPFP†	421–430
ZNP41-2-4		
ZNP74-7	YDDDDDIPFPGPINDDDNPG	421–440
ZNP24-4-2	QTQFRPIQNVPGPHRTIHHA	521–540
	TPTVAPPAPVYRDHSEKKEL	601–620
ZNP106-9	DTTIPDVVVD†	451–460
ZNP98-7	MLTPINEEADPLDDADDETS	561–580
ZNP35-16-3-5	DDEDTKPVPNRSTKGGQQKN	491–510
ZNP62-7	YRDHSEKKELPQDEQQDQDH	611–630

†: Overlapping sequence of two consecutive peptides to which the antibodies bound.

Three highly cross-reactive mAbs, ZNP41-2-4, ZNP31-1-8 and ZNP74-7, strongly reacted to the peptide corresponding to aa positions 421-440. ZNP41-2-4 and ZNP31-1-8 reacted further with the consecutive peptides corresponding to aa positions 411-430, restricting the recognized epitope to ten aa (aa positions 421-430). Another cross-reactive mAb, ZNP24-4-2, bound to two peptides corresponding to very different regions in NP. ZNP106-9 reacted with two consecutive peptides with overlapping aa sequences corresponding to aa positions 441-

460 and 451-470, sharing the ten aa at positions 451–460. ZNP98-7, ZNP35-16-3-5 and ZNP62-7 each recognised a single peptide derived from different regions of NP (aa 561–580, aa 491–510 and aa 611–630, respectively). Figure 4-6 shows the epitope locations in the schematic diagram of NP.

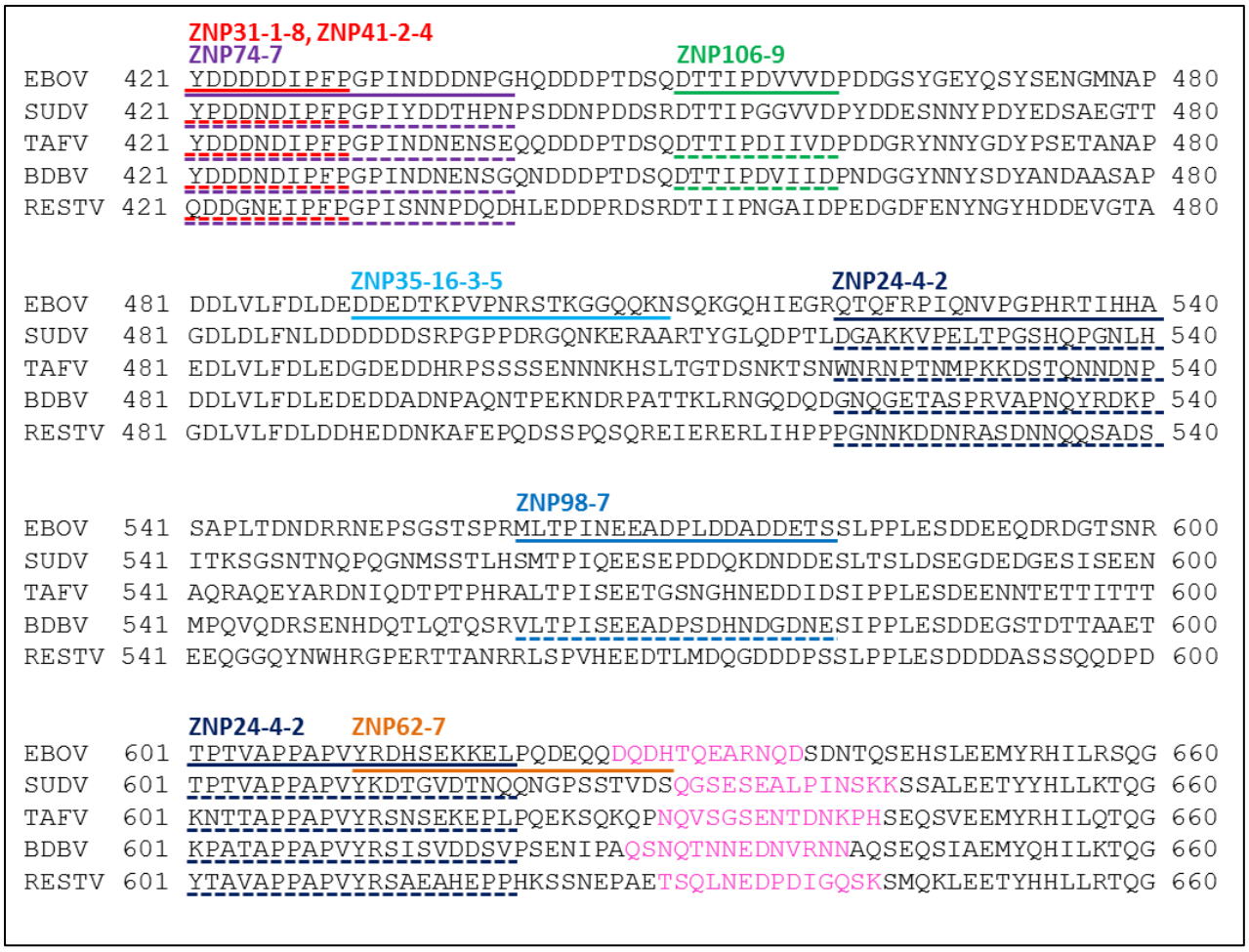

Figure 4-5: Location of epitope peptide sequences. Amino acid sequences of EBOV, SUDV, TAFV, BDBV and RESTV were obtained from GenBank under accession numbers AF272001, AF173836, FJ217162, FJ217161 and AF522874, respectively. Amino acid sequences at positions 421-660 of each virus are shown. EBOV NP peptides recognized by the mAbs are highlighted with solid lines. Corresponding regions of the other NPs to which each mAb showed strong cross-reactivity are underlined (dashed lines). Amino acid sequences used for producing species-specific rabbit antisera are shown in pink.

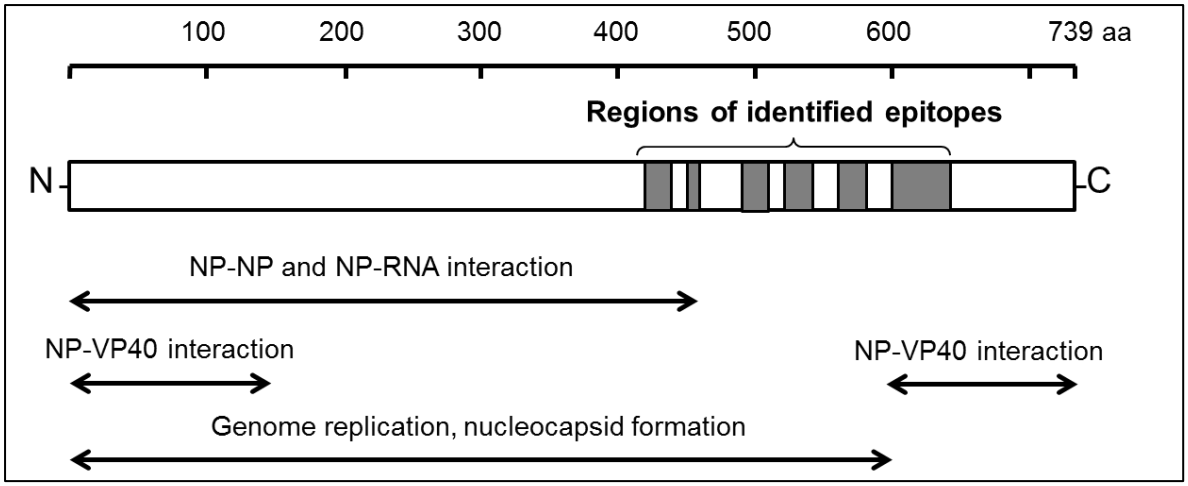

Figure 4-6: Locations of the identified epitopes (grey) are shown in the schematic diagram of NP. Functional domains (Watanabe *et al*., 2006; Noda *et al*. 2007; Bharat *et al*., 2012) are also shown.

4.4 Reactivity of rabbit antisera produced by immunisation with synthetic peptides.

Determination of epitopes distinctive among the NPs of each filovirus species belonging to the genus *Ebolavirus* and *Marburgvirus* was attempted. Based on a program used to predict B-cell epitopes, the region around aa positions 630-650 from viruses representing each filovirus species (Fig. 4-5) (EBOV, SUDV, TAFV, BDBV, RESTV and MARV) was selected, and rabbit antisera to the respective synthetic peptides were generated. The reactivity of each antiserum (FS0169, FS0191, FS0046, FS0048, FS0170 and FS0610) was analysed by ELISA (Table 4-3). According to the high sequence variation in this region among these viruses, the antisera reacted specifically with the homologous NPs, although FS0046 and FS0048 (antisera to TAFV and BDBV, respectively), showed limited cross-reactivity to RESTV NP. The virus specificity was further confirmed using filovirus lysates in Western blot analysis (Fig. 4-7). Notably, all the virus strains tested within the genus *Marburgvirus* (including RAVV) were recognized by antiserum FS0610. These results

indicated that the region around aa 630-650 in filovirus NP served as a filovirus species-specific epitope.

Table 4-3: Reactivity of rabbit antisera produced by immunisation with synthetic peptides.

Antiserum	Synthetic peptide used for immunisation (amino acid sequence)	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
FS0169	EBOV NP 628-638 (QDHTQEARNQD)	++*	-	-	-	-	-
FS0191	SUDV NP 631-644 (QGSESEALPINSKK)	-	++	-	-	-	-
FS0046	TAFV NP 630-643 (NQVSGSENTDNKPH)	-	-	++	-	+	-
FS0048	BDBV NP 628-641 (QSNQTNNEDNVRNN)	-	-	-	++	+	-
FS0170	RESTV NP 630-643 (TSQLNEDPDIGQSK)	-	-	-	-	+	-
FS0610	MARV NP 635-652 (RVVTKKGRTFLYPNDLLQ)	-	-	-	-	-	++

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450} values at a serum dilution of 1:2,000. ++: OD ≥ 1.0; +: 0.5 < OD < 1.0; -: OD ≤ 0.5.

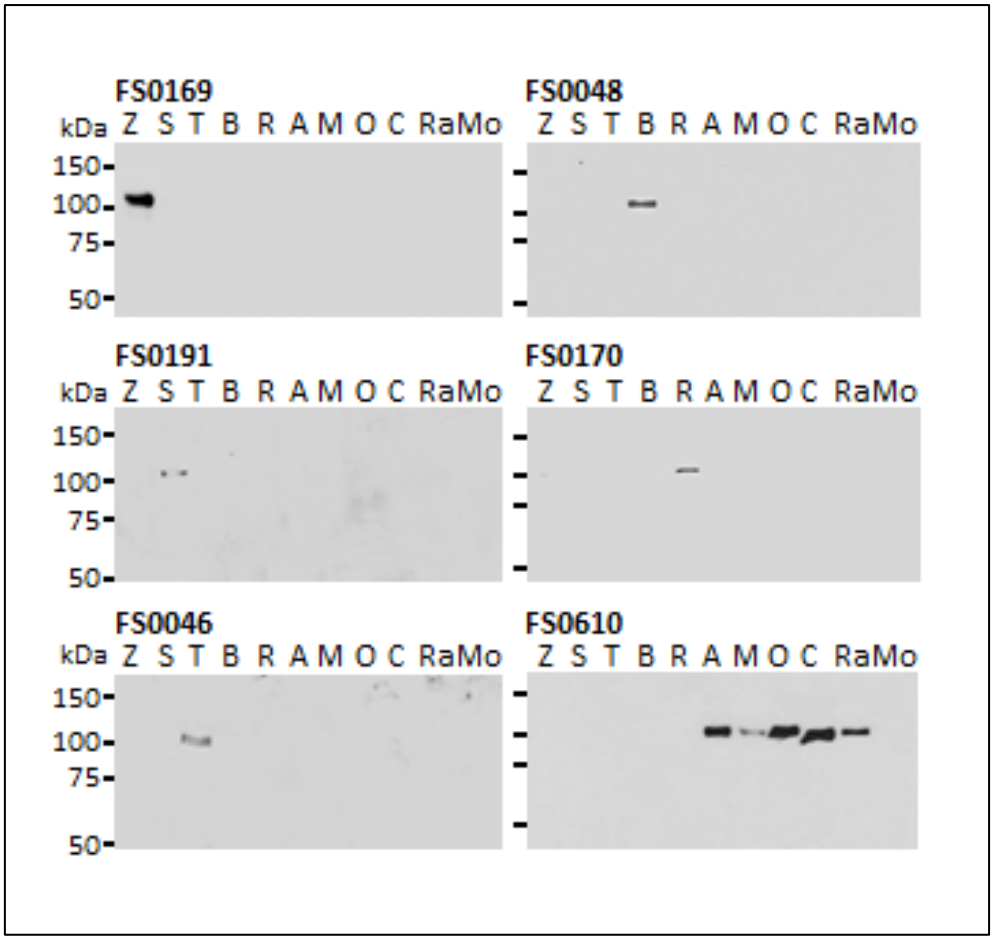

Figure 4-7: Reactivity of rabbit antisera in Western blot analysis. Rabbit antisera (FS0169, FS0191, FS0046, FS0048, FS0170 and FS0610) were produced using synthetic peptides derived from EBOV, SUDV, TAFV, BDBV, RESTV and MARV, respectively. Experimental conditions were the same as in figure 4-3. EBOV (Z), SUDV (S), TAFV (T), BDBV (B), RESTV (R), MARV Angola (A), MARV Musoke (M), MARV Ozolin (O), MARV Ci67 (C), RAVV (Ra). Mo, mock-infected.

4.5 Immunochromatographic strip test

4.5.1 Reactivity of latex conjugated mAbs tested

To prepare the test device, mAb cross-reactive to NPs of all known ebolaviruses were first selected and the reactivity of these mAbs conjugated with latex was analysed. Only ZNP31-1-8 gave a strong positive reaction to EBOV, while ZNP74-7 had a weak reaction (Table 4-

4). The ZNP41-2-4 did not react at all. Consequently, ZNP31-1-8 was used to prepare the immunochromatographic strip to detect the ebolavirus NP antigen.

Table 4-4: Reactivity of different latex conjugated mAbs.

	EBOV	SUDV	TAFV	BDBV	RESTV
MGP14-22	-	NT	NT	NT	NT
ZGP42/3.7	-	NT	NT	NT	NT
ZNP31-1-8	+	+/-	+	+	+/-
ZNP41-2-4	-	NT	NT	NT	NT
ZNP74-7	+/-	NT	NT	NT	NT

+: strong positive; +/-: weak positive; -: negative; NT: not tested

4.5.2 Reactivity of the immunochromatographic strip test to VLPs and authentic virus lysates

The test device based on the immunochromatographic strip was prepared and its specificity and sensitivity were evaluated using VLPs (Fig. 4-8). The test device was able to positively detect the NP antigens in VLPs derived from EBOV, BDBV and TAFV proteins, but unable to detect NPs in SUDV and BDBV VLPs (Table 4-5).

Figure 4-8: Immunochromatographic strip test positive (a) and negative (b) windows

Table 4-5: Reactivity of VLPs on immunochromatographic strip test

Filovirus VLPs	Result
EBOV	+
SUDV	-
TAFV	+
BDBV	+
MARV	-
LLOV	-
PCAGGS	-

+: positive; -: negative. MARV, LLOV and PCAGGS plasmid were used as negative controls.

When tested with authentic ebolavirus in the supernatants of infected Vero E6 cells, there was no reaction at all for any of the viruses (Table 4-6).

Table 4-6: Reactivity of authentic filoviruses on immunochromatographic strip test

Authentic filovirus	Result
EBOV	-
SUDV	-
TAFV	-
BDBV	-
MARV	-

+: positive; -: negative. MARV was used as a negative control.

CHAPTER FIVE

5.0 DISCUSSION

In this study, mAbs to ebolavirus NP were produced and characterised. A selected mAb was then used to develop an immunochromatographic strip test for identification of ebolavirus infections.

Of the initial 127 hybridomas producing ZNP antibodies (Appendix A), there were five that were cross-reactive to all ebolaviruses. Three of these with the highest ELISA OD_{450} value, ZNP31, ZNP41 and ZNP74, were selected for cloning for production of mAbs, resulting in the production of ZNP31-1-8, ZNP41-2-4 and ZNP74-7, respectively. These mAbs, cross reactive to all known ebolaviruses, were very important for the development of this immunochromatographic strip test. This is the first report of mAbs that can detect all known ebolaviruses. These mAbs also have the potential for identification of as yet unknown *Ebolavirus* species. During the first reported outbreak of BDBV, initial diagnosis was made using an antigen capture ELISA using a combination of anti- EBOV, SUDV and RESTV mAbs as well as hyper-immune polyclonal anti-EBOV rabbit serum, whereas the specimen were initially negative when tested by RT-PCR (Towner *et al*., 2008). This highlights the value of mAbs to potentially identify new *Ebolavirus* species.

On Western blotting analysis using lysates of actual filovirus particles grown in Vero E6 cells (Fig. 4-3), it was found that the mAbs predominantly bound to proteins of approximately 100 kDa and some smaller proteins, representing full-length NP and likely degraded NP molecules, respectively (Watanabe *et al*., 2006). The cross-reactivity profiles and virus specificities were similar to those obtained by ELISA, thus confirming the utility of these mAbs to detect not only recombinant but also virus-derived NPs. The apparent change in

cross-reactivity of some of the mAbs after cloning was probably because the initial hybridomas comprised various cell populations producing several different antibodies with different reactivities, which could have diluted the effect of that specific hybridoma cell.

Using mouse mAbs and synthetic peptide-based scanning, two highly conserved antigenic regions were determined (aa 421-440 and aa 601-620) serving as linear epitopes in the filovirus NP (Fig. 4-5). In addition, a stretch of ten aa at aa 421-430 (YDDDDDIPFP) was found to be important for three mAbs (ZNP31-1-8, ZNP41-2-4 and ZNP74-7), which strongly recognized all known viruses in the genus *Ebolavirus*. This finding is consistent with a previous study demonstrating that mAbs reactive to EBOV, RESTV and SUDV recognised the sequence at aa 424-430 (Niikura *et al*., 2003). In this specific region, the aa sequence $I_{427}PFP_{430}$ is completely conserved among all analysed viruses in the *Ebolavirus* genus, suggesting that these aa residues are crucial for conformation of this common epitope.

The mAb ZNP24-4-2 was highly cross-reactive to all known viruses of the genus *Ebolavirus*, with weaker reactivity to SUDV (Table 4-1 and Fig. 4-3). This mAb reacted with two different peptides corresponding to aa 521-540 and aa 601-620 (Fig. 4-5). These two peptide sequences may be parts of a conformational epitope. However, there is no conserved sequence in the region at aa 521-540 among all the analysed viruses, whereas the sequence at aa 601-620 shows some conservation. Although SUDV was only weakly recognised by this mAb, this conserved region might be required for recognition as a conformational epitope.

The mAbs ZNP106-9 and ZNP108-2-5 were strongly reactive to EBOV, TAFV and BDBV, but only weakly reactive or nonreactive to SUDV and RESTV, respectively. This reactivity pattern is consistent with the phylogenetic relationship among the viruses (Towner *et al*., 2008). Only mAb ZNP106-9 reacted with the peptide sequence D_{451}TTIPDVVVD$_{460}$, demonstrating that ZNP108-2-5 recognises a different epitope. The aa sequence alignment of

this region suggests that D_{456} in EBOV, TAFV and BDBV is critical for the mAb ZNP106-9 specificity to these viruses, since SUDV and RESTV have G or N at this aa position, respectively (Fig. 4-5).

The mAbs ZNP35-16-3-5 and ZNP62-7 recognised EBOV only, and bound to aa 611-630 and aa 491-510, respectively. According to the sequence variation among the analysed viruses, these aa likely form EBOV-specific epitopes. It can be speculated that the same region of NP of the other viruses in the *Ebolavirus* genus forms species-specific epitopes. In addition to these two regions, the success of the production of antisera to the synthetic peptides with the predicted sequences around aa 630-650 provided further information on the filovirus species-specific epitopes. Antisera to TAFV and BDBV (FS0046 and FS0048 respectively), showed limited cross-reactivity to RESTV NP. A comparison of the respective aa sequences shows that BDBV and RESTV had the same aa sequence for aa $N_{634}ED_{636}$, which probably accounts for the cross-reactivity. While no such similarities were found between TAFV and RESTV, some of the aa were similar in that they were hydrophobic e.g. aa $N_{630}Q_{631}$ (TAFV) and $T_{630}S_{631}$ (RESTV) and $S_{634}GS_{636}$ (TAFV) and $G_{640}QS_{642}$ (RESTV).

The mAbs ZNP 98-7 and 105-7 did not show any reactivity on PepScreen ELISA. This was most probably because they recognised conformational epitopes and not linear epitopes.

The antigenic region of EBOV NP was previously shown to be located in the C-terminal half of the protein (Saijo *et al*., 2001a). The N-terminal aa 1-451 of the EBOV NP assemble into a condensed helix, which forms the inner structure of the viral nucleocapsid (Bharat *et al*., 2012). The aa residues in this region are highly conserved among the known viruses in the genus *Ebolavirus*. It is likely that this region forms functionally important structures inside the NP molecule, and as a result, has limited antigenic properties. This is consistent with our results in which most antigenic regions were found in the highly variable C-terminal region

starting at aa 451 (Fig. 4-5). The only epitope found on the condensed helix structure was the one recognised by ZNP31-1-8, ZNP41-2-4 and ZNP74-7, mAbs cross-reactive to all known *Ebolavirus* species.

This is the first report of an immunochromatographic strip test developed for detection of filoviral infection. This type of test would especially be of great significance in large outbreaks, to provide cheap, rapid diagnosis of infection, as well as in inter-outbreak periods for monitoring and surveillance. Currently, available tests are expensive to run, requiring specialised training and equipment and for some tests BSL-4 containment laboratory is required (Wang *et al*., 2011). In some instances, specimen have to be transported to other countries for confirmatory diagnosis, resulting in a lag of implementation of outbreak measures (MacNeil *et al*., 2011a).

The immunochromatographic strip test produced in this study was able to detect EBOV, TAFV and BDBV VLPs but not able to detect SUDV and RESTV VLPs. This may be due to their phylogenetic relationship (Towner *et al*., 2008). Looking at the epitope sequence recognised by this mAb, SUDV and RESTV had some differences. For SUDV instead of D_{422}, there was P_{422}, while for RESTV, instead of Y_{421}, D_{424} and D_{426} there was Q_{421}, G_{424} and E_{426}, respectively. The immunochromatographic strip test was not able to detect authentic viruses. This could be as a result of inherent low sensitivity of the test. Another contributing factor could be the concentration of the antigen within the sample, as sensitivity of immunochromatographic strip tests is directly correlated to the pathogen load in the sample (Moore, 2013).

The mAb used on this test was ZNP31-1-8 which was cross reactive to all known ebolaviruses belonging to the different species on ELISA and Western blot analysis of live virus. Sensitivity of ELISA tests often do not correlate with those on

immunochromatographic strip tests as seen in studies comparing ELISA and immunochromatographic strip tests for the detection of Dengue virus (Dussart *et al*., 2008; Hang *et al*., 2009; Ramirez *et al*., 2009; Najioullah *et al*., 2011). One reason could be the effect of the different 'solid phases' of the tests, i.e. ELISA used a plastic polymer plate, while the immunochromatographic strip test used a nitrocellulose membrane. This could also be due to a loss of structural integrity of the mAb after adsorption onto the nitrocellulose membrane. Ideally after adsorption, it must retain its structural integrity when dried and be instantly reactive when rehydrated by the sample (Millipore, 2008). Loss of structural integrity may also result from the conjugation of the mAb to the latex beads (Siiman *et al*., 2001) as well as changes in pH or salt concentration (Lipman *et al*., 2005).

The sensitivity of the test has to be increased before it can undergo validation and become commercially available. Considering the need for improvement of the sensitivity of the test, optimisation of factors like the pH of the solution used to immobilise the mAbs to the membrane as well as the membrane pore size should be conducted. Other factors to be evaluated include different mAb concentrations, quality and concentration of the latex particles, and optimum sample volume (Fernandez-Sanchez *et al*., 2005; Safenkova *et al*., 2012). To further improve the sensitivity, different sample buffers can be evaluated. A change in buffers resulted in improved test sample migration as well as a stronger positive test reaction for an immunochromatographic test for foot and mouth disease (Ferris *et al*., 2009). The test line could also be positioned further downstream to enhance the interaction time between the mAbs and the antigen (Posthuma-Trumpie *et al*., 2009). Several different mAbs could be pooled to increase sensitivity (Lipman *et al*., 2005; Bruning-Richardson *et al*., 2011). The following combinations could be attempted in addition to using ZNP31-1-8: ZNP24-4-2 and ZNP106-9 for SUDV and BDBV; ZNP105-7 for TAFV, BDBV and RESTV.

Furthermore, other mAbs from the repertoire of 127 hybridomas produced in this study can be used.

Immunochromatographic strip tests, using mAbs to detect viral antigen, have been successfully developed for a number of viral diseases. Clearview^{TM} was developed with a single mAb and has been used for detection of rinderpest in the field. Although its sensitivity compared to that of RT-PCR is low, it can identify positive herds (Bruning *et al*., 1999). This demonstrates that even with lower sensitivity than conventional tests like RT-PCR, immunochromatographic strip tests can still be used for diagnosis in the field. Subsequently, an improved test for rinderpest, using two mAbs with a resultant higher detection limit than Clearview^{TM} was developed (Bruning-Richardson *et al*., 2011), suggesting that using a combination of mAbs can enhance the sensitivity of an immunochromatographic test.

Once validated this test could prove to be a useful tool in the diagnosis and surveillance of ebolavirus infections, especially in resource-poor areas with inadequate or non-existent diagnostic facilities. In the event of a large outbreak similar to the current situation in west Africa, the test could be used for rapid screening of populations in affected areas, as well as those individuals travelling from affected to non-affected areas. Ideally the test will be able to diagnose all infections caused by all species of *Ebolavirus*, known and unknown. This test is simple to perform, robust with no special storage or handling conditions and rapid, taking only 20 minutes to diagnosis. This will lead to a potential reduction of the time taken from the initial outbreak case to a confirmatory diagnosis, resulting in limited to no secondary transmission, thereby contributing to better disease outbreak preparedness.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

In this study, a panel of NP-specific mAbs divided into seven groups based on their cross-reactivity profiles to all known viruses of the genus *Ebolavirus* was established. Using synthetic peptide-based screening, eight antigenic regions in the EBOV NP molecule, each consisting of roughly ten to 20 aa residues, were determined. Three mAbs were able to detect all known *Ebolavirus* species when used in ELISA and Western blot tests. This is the first report of production of mAbs that can detect all known ebolaviruses. One of these three mAbs, ZNP31-1-8, was selected for development of the first immunochromatographic strip test for detection of filoviral infection. This test was able to detect EBOV, BDBV and TAFV VLPs. This test has great potential as a rapid detection test for diagnosis as well as monitoring and surveillance studies. Further work needs to be done to optimise the test for detection of VLPs and authentic viruses before it can undergo laboratory and field validation.

6.2 Recommendations

Considering the successful production and characterisation of mAbs to ebolavirus NP, and their use in the development of an immunochromatographic strip test, the following recommendations are made:

1. Once the test sensitivity has been optimised, laboratory validation of the test should be conducted, in comparison to conventional methods of diagnosis of ebolavirus infections, namely ELISA, RT-PCR and virus isolation by cell culture. This should be followed by field validation.

2. Once validated, health policy makers in government and the World Health Organisation should be encouraged to consider it for rapid diagnosis of ebolavirus infections.
3. The mAbs produced in this study have potential use in the development of new generation diagnostic tests based on ELISA and immunohistochemistry for the detection of ebolavirus antigen.

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APPENDICES

Appendix A: Cross-reactivity profiles to all known *Ebolavirus* species (EBOV, SUDV, TAFV, BDBV and RESTV), and Marburg virus (MARV) of antibodies secreted by hybridomas.

Table 1: Antibodies only recognising EBOV

Sample#	EBOV[†]	SUDV	TAFV	BDBV	RESTV	MARV
ZNP1	++[*]	-	-	-	-	-
ZNP6	++	-	-	-	-	-
ZNP8	++	-	-	-	-	-
ZNP9	++	-	-	-	-	-
ZNP10	++	-	-	-	-	-
ZNP12	++	-	-	-	-	-
ZNP13	++	-	-	-	-	-
ZNP16	++	-	-	-	-	-
ZNP17	++	-	-	-	-	-
ZNP18	++	-	-	-	-	-
ZNP21	++	-	-	-	-	-
ZNP27	++	-	-	-	-	-
ZNP29	++	-	-	-	-	-
ZNP35	++	-	-	-	-	-
ZNP36	++	-	-	-	-	-
ZNP37	++	-	-	-	-	-
ZNP38	++	-	-	-	-	-
ZNP40	++	-	-	-	-	-
ZNP42	++	-	-	-	-	-
ZNP44	++	-	-	-	-	-
ZNP46	++	-	-	-	-	-
ZNP47	++	-	-	-	-	-

Sample#	EBOV[†]	SUDV	TAFV	BDBV	RESTV	MARV
ZNP49	++	-	-	-	-	-
ZNP51	++	-	-	-	-	-
ZNP53	++	-	-	-	-	-
ZNP54	++	-	-	-	-	-
ZNP55	++	-	-	-	-	-
ZNP56	++	-	-	-	-	-
ZNP61	++	-	-	-	-	-
ZNP62	++	-	-	-	-	-
ZNP65	++	-	-	-	-	-
ZNP69	++	-	-	-	-	-
ZNP72	++	-	-	-	-	-
ZNP79	++	-	-	-	-	-
ZNP82	++	-	-	-	-	-
ZNP84	+	-	-	-	-	-
ZNP87	++	-	-	-	-	-
ZNP88	++	-	-	-	-	-
ZNP93	++	-	-	-	-	-
ZNP95	++	-	-	-	-	-
ZNP100	++	-	-	-	-	-
ZNP103	++	-	-	-	-	-
ZNP104	++	-	-	-	-	-
ZNP111	++	-	-	-	-	-
ZNP122	++	-	-	-	-	-
ZNP123	++	-	-	-	-	-

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
ZNP124	++	-	-	-	-	-
ZNP126	++	-	-	-	-	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: OD ≥ 1.0; +: 0.3 < OD < 1; -: OD ≤ 0.3.

Table 2: Antibodies recognising EBOV, SUDV, TAFV, BDBV and RESTV

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
ZNP20	++*	+	+	++	++	-
ZNP26	++	++	++	++	++	-
ZNP28	++	++	+	++	++	-
ZNP31	++	++	++	++	++	-
ZNP34	++	+	++	+	+	-
ZNP41	++	++	++	++	++	-
ZNP74	++	++	++	++	++	-
ZNP77	++	+	+	++	+	-
ZNP85	++	+	+	++	++	-
ZNP91	++	++	++	+	+	-
ZNP119	++	++	++	++	++	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: OD ≥ 1.0; +: 0.3 < OD < 1.0; -: OD ≤ 0.3.

Table 3: Antibodies recognising EBOV, SUDV, BDBV and RESTV

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
ZNP19	++*	++	-	+	++	-
ZNP116	++	++	-	+	++	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: OD ≥ 1.0; +: 0.3 < OD < 1.0; -: OD ≤ 0.3.

Table 4: Antibodies recognising EBOV, BDBV and RESTV

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
ZNP7	++*	-	-	++	+	-
ZNP86	++	-	-	+	+	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: OD ≥ 1.0; +: 0.3 < OD < 1.0; -: OD ≤ 0.3.

Table5: Antibodies recognising EBOV and RESTV

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
ZNP105	++*	-	-	-	+	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: OD ≥ 1.0; +: 0.3 < OD < 1.0; -: OD ≤ 0.3.

Table 6: Antibodies recognising EBOV, BDBV and TAFV

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
ZNP2	++*	-	+	++	-	-
ZNP3	++	-	+	+	-	-
ZNP23	++	-	++	++	-	-
ZNP25	++	-	++	+	-	-
ZNP39	++	-	++	++	-	-
ZNP59	++	-	++	+	-	-
ZNP64	++	-	+	+	-	-
ZNP83	++	-	++	+	-	-
ZNP94	++	-	++	+	-	-
ZNP106	++	-	+	++	-	-
ZNP107	++	-	+	++	-	-
ZNP112	++	-	++	++	-	-
ZNP115	++	-	++	+	-	-
ZNP118	++	-	+	+	-	-
ZNP120	+	-	+	+	-	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: $OD \geq 1.0$; +: $0.3 < OD < 1.0$; -: $OD \leq 0.3$.

Table 7: Antibodies recognising EBOV, TAFV, BDBV and RESTV

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
ZNP4	++*	-	++	+	++	-
ZNP5	++	-	+	+	+	-
ZNP22	++	-	++	+	++	-
ZNP24	++	-	++	++	++	-
ZNP33	++	-	++	+	++	-
ZNP45	++	-	++	+	++	-
ZNP48	++	-	++	+	++	-
ZNP52	++	-	++	+	++	-
ZNP58	++	-	++	+	++	-
ZNP80	++	-	++	+	++	-
ZNP81	++	-	++	+	++	-
ZNP97	++	-	++	++	++	-
ZNP99	++	-	++	+	++	-
ZNP109	++	-	+	++	+	-
ZNP113	++	-	++	+	++	-
ZNP117	++	-	++	+	++	-
ZNP121	++	-	++	+	++	-
ZNP127	++	-	++	+	++	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: $OD \geq 1.0$; +: $0.3 < OD < 1.0$; -: $OD \leq 0.3$.

Table 8: Antibodies recognising EBOV, TAFV and RESTV

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
ZNP14	++*	-	+	-	++	-
ZNP67	++	-	++	-	+	-
ZNP71	++	-	++	-	++	-
ZNP73	++	-	++	-	++	-
ZNP89	++	-	+	-	++	-
ZNP92	++	-	+	-	+	-
ZNP101	++	-	+	-	+	-
ZNP110	++	-	+	-	++	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: OD ≥ 1.0; +: 0.3 < OD < 1.0; -: OD ≤ 0.3.

T**able 9: Antibodies recognising EBOV, SUDV, TAFV and RESTV**

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
ZNP70	++*	++	+	-	+	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: OD ≥ 1.0; +: 0.3 < OD < 1.0; -: OD ≤ 0.3.

Table 10: Antibodies recognising EBOV and BDBV

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
98	++*	-	-	+	-	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: OD ≥ 1.0; +: 0.3 < OD < 1.0; -: OD ≤ 0.3.

Table 11: Antibodies recognising EBOV, SUDV, TAFV and BDBV

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
11	++*	+	++	++	-	-
102	++	+	+	+	-	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: OD ≥ 1.0; +: 0.3 < OD < 1.0; -: OD ≤ 0.3.

Table 11: Antibodies recognising EBOV and TAFV

Sample#	EBOV†	SUDV	TAFV	BDBV	RESTV	MARV
15	++*	-	++	-	-	-
76	++	-	+	-	-	-
108	++	-	++	-	-	-

†: VLPs of each virus species were used as antigens. *: Antibody reactivity was evaluated based on ELISA OD_{450}. ++: OD ≥ 1.0; +: 0.3 < OD < 1.0; -: OD ≤ 0.3.

Appendix B: Reprint of published material

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Mapping of conserved and species-specific antibody epitopes on the Ebola virus nucleoprotein

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ABSTRACT

Filoviruses (viruses in the genus *Ebolavirus* and *Marburgvirus* in the family *Filoviridae*) cause severe haemorrhagic fever in humans and nonhuman primates. Rapid, highly sensitive, and reliable filovirus-specific assays are required for diagnostics and outbreak control. Characterisation of antigenic sites in viral proteins can aid in the development of viral antigen detection assays such immunochromatography-based rapid diagnosis. We generated a panel of mouse monoclonal antibodies (mAbs) to the nucleoprotein (NP) of Ebola virus belonging to the species *Zaire ebolavirus*. The mAbs were divided into seven groups based on the profiles of their specificity and cross-reactivity to other species in the *Ebolavirus* genus. Using synthetic peptides corresponding to the Ebola virus NP sequence, the mAb binding sites were mapped to seven antigenic regions in the C-terminal half of the NP, including two highly conserved regions among all five *Ebolavirus* species currently known. Furthermore, we successfully produced species-specific rabbit antisera to synthetic peptides predicted to represent unique filovirus B-cell epitopes. Our data provide useful information for the development of Ebola virus antigen detection assays.

1. Introduction

Filoviruses are among the most lethal human pathogens recognised to date with case fatality rates up to 90%, depending on the virus species and strain (Pittalis et al., 2009; Bente et al., 2009). Filoviruses are grouped into two genera, *Ebolavirus* and *Marburgvirus*. There is one known species of *Marburgvirus*, *Marburg marburgvirus*, consisting of two viruses, Marburg virus (MARV) and Ravn virus (RAVV). In contrast, the genus *Ebolavirus* has five known species, *Zaire ebolavirus*, *Sudan ebolavirus*, *Taï Forest ebolavirus*, *Bundibugyo ebolavirus* and *Reston ebolavirus*, represented by Ebola virus (EBOV), Sudan virus (SUDV), Taï Forest virus (TAFV), Bundibugyo virus (BDBV) and Reston virus (RESTV), respectively. Furthermore, there is a newly discovered filovirus named Lloviu virus (LLOV) assigned to the proposed genus *Cuevavirus*, with one species, *Lloviu cuevavirus* (Negredo et al., 2011; Kuhn et al., 2010). The genome of filoviruses is approximately 19 kb long, and contains seven genes arranged sequentially in the order: nucleoprotein (NP), viral protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24 and polymerase (L) genes (Sanchez et al., 2007).

The lack of therapeutics and vaccines for filovirus infections and the fact that other pathogens cause clinical symptoms comparable to those of Ebola and Marburg haemorrhagic fever highlights the need for rapid, sensitive, reliable and virus-specific diagnostic tests to control the spread of these viruses (Qiu et al., 2011; Sanchez et al., 2007). Rapid antigen-detection tests with filovirus-specific monoclonal antibodies (mAb) are likely one of the best ways for early diagnosis of filovirus infections in the field setting. NP may be the ideal target antigen because of its abundance in filovirus particles and its strong antigenicity (Niikura et al., 2001, 2003). The average EBOV virion, which is up to 1028 nm in length, contains about 3200 NP molecules (Bharat et al., 2012). EBOV NP consists of 739 amino acid residues, with a conserved hydrophobic N-terminus and a variable hydrophilic C-terminal part (Niikura et al., 2001;

Abbreviations: mAb, monoclonal antibodies; EBOV, Ebola virus; SUDV, Sudan virus; TAFV, Taï Forest virus; BDBV, Bundibugyo virus; RESTV, Reston virus; MARV, Marburg virus; RAVV, Ravn virus; NP, nucleoprotein; VP, viral protein; GP, glycoprotein; VLP, virus-like particle.

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Sanchez et al., 2007). NP plays an important role in the replication of the viral genome and is essential for formation of the nucleocapsid (Watanabe et al., 2006). The C-terminus of EBOV NP binds to VP40 while the N-terminus forms a condensed helix with the same diameter as the inner nucleocapsid helix of an EBOV particle (Bharat et al., 2012). Following expression of VP40 in cultured cells, virus-like particles (VLPs) are produced and, upon co-expression of NP, the VLP contains NP as its core (Bharat et al., 2012; Noda et al., 2007). It has been demonstrated that the C-terminal half of the filovirus NP has strong antigenicity (Saijo et al., 2001). Multiple studies have identified conformational and linear epitopes for antibodies in this NP region for several viruses within the genus *Ebolavirus* (Ikegami et al., 2003; Niikura et al., 2001, 2003).

In general, characterisation of antigenic sites in a viral protein can aid in the development of diagnostic tools, therapeutics and vaccines (Gershoni et al., 2007; Toyoda et al., 2000). Here, we identified antigenic regions within the NP molecule using mouse NP-specific mAbs and rabbit antisera to synthetic NP peptides representing viruses from all known filovirus species. Some of the identified antigenic regions are shared among multiple virus species within the *Ebolavirus* genus, whereas others are species-specific. Our data provide useful information for future development of antigen-based detection assays for the diagnosis of filovirus infections.

2. Materials and methods

2.1. Plasmid construction

Plasmids expressing GP, VP40 and NP were constructed as described previously (Nakayama et al., 2010; Nidom et al., 2012). Briefly, viral RNAs were extracted from the supernatant of Vero E6 cells infected with EBOV (Mayinga), SUDV (Boniface), TAFV (Côte d'Ivoire), BDBV (Bundibugyo), RESTV (Pennsylvania) or MARV (Angola). Full length NP, VP40 and GP cDNA were amplified by RT-PCR using KOD-plus-Neo polymerase (Toyobo) and cloned into TOPO® vector using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen). After sequence confirmation, the cloned genes were inserted into the mammalian expression vector pCAGGS.

2.2. Preparation of purified VLPs and NP

Human epithelial kidney 293T cells were grown in Dulbeco's modified Eagle's medium (DMEM), supplemented with 10% FCS, penicillin (100 unit/ml) and streptomycin (100 μg/ml). VLPs were produced by transfection of 293T cells with plasmids expressing NP and VP40 together with or without the plasmid expressing GP as described previously (Licata et al., 2004; Urata et al., 2007). Forty-eight hours after transfection, VLPs in the supernatant were purified by centrifugation through a 25% sucrose cushion at 28,000 × *g* and 4 °C for 1.5 h. The pelleted VLPs were resuspended in PBS and stored at −80 °C. For the preparation of purified NP, 293 T cells transfected with the plasmid encoding EBOV NP were lysed, and the NP fraction was collected by discontinuous CsCl gradient centrifugation of the lysates as described previously (Bharat et al., 2012; Noda et al., 2010).

2.3. Mouse mAb production

On day 0, six-week-old female Balb/c mice were immunised intramuscularly with 100 μg of EBOV VLPs consisting of NP and VP40 with complete Freund's adjuvant (Difco). The animals were boosted intramuscularly on day 14 with 100 μg of the same EBOV VLPs and incomplete Freund's adjuvant. After a final intravenous boost with 100 μg of the same EBOV VLPs without adjuvant on day 39, spleen cells were harvested on day 42 and fused to P3-U1 myeloma cells according to standard procedures (Shahhosseini et al., 2007). Hybridomas were maintained in Roswell Park Memorial Institute medium 1640 containing 20% FCS, penicillin (100 unit/ml), streptomycin (100 μg/ml), L-glutamine (4 mM) and 2-mercaptoethanol (55 μM). Hybridoma supernatants were screened by an enzyme linked immunosorbent assay (ELISA) for secretion of NP-specific antibodies using purified EBOV NP and VLP as antigens. Specificity and cross-reactivity of mAbs were also confirmed by Western blotting. Selected hybridoma cells were then cloned twice performing limiting dilution.

2.4. Production of rabbit antisera

Genetyx ver6.0 for Windows (GENETYX CORPORATION) was used to predict B-cell epitopes in the NPs of EBOV, SUDV, TAFV, BDBV, RESTV and MARV, and the amino acid (aa) positions around 630–650 were selected. Synthetic peptides corresponding to this aa region in NP were produced (Sigma). Rabbits were then immunised with keyhole limpet haemocyanin-conjugated synthetic peptides by the standard procedure, and antisera were obtained on day 49.

2.5. ELISA

Ninety six-well ELISA plates (Nunc®, Maxisorp) were coated with 50 μl PBS containing purified EBOV NP (2 μg/ml), VLPs (2–5 μg/ml) or synthetic peptides (100 μg/ml) per well overnight at 4 °C. ELISA was carried out as described previously (Nakayama et al., 2011), using mouse antisera, hybridoma supernatants, purified mAbs or rabbit antisera as primary antibodies and goat anti-mouse IgG (H + L) or donkey anti-rabbit IgG (H + L) conjugated with peroxidase (Jackson ImmunoResearch) as secondary antibodies.

2.6. Western blotting

Vero E6 cells cultured in DMEM supplemented with 10% FBS, penicillin (100 unit/ml), streptomycin (100 μg/ml) and L-glutamine (4 mM) were infected with EBOV (Mayinga), SUDV (Boniface), TAFV (Cote d'Ivoire), BDBV (Bundibugyo), RESTV (Pennsylvania), MARV (Angola, Musoke, Ozolin and Ci67) or RAVV (Ravn) at a multiplicity of infection of 1 and maintained for 72 h. Cell culture supernatants were subjected to SDS-PAGE. For the screening of hybridoma supernatants (see above), VLPs were used instead of authentic virus lysates. After electrophoresis, separated proteins were blotted on a polyvinylidene difluoride membrane (Millipore) or Immobilon-P transfer membrane (Millipore). Mouse mAbs and rabbit antisera were used as primary antibodies. The bound antibodies were detected with peroxidase-conjugated goat anti-mouse IgG (H + L) or donkey anti-rabbit IgG (H + L) (Jackson ImmunoResearch), followed by visualisation with Immobilon Western (Millipore).

2.7. Ethics and biocontainment statements

Animal studies were carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The protocol was approved by the Hokkaido University Animal Care and Use Committee. All efforts were made to minimise the suffering of animals. All infectious work with filoviruses was performed under high containment complying with standard operating procedures approved by the Institutional Biosafety Committee in the BSL4 Laboratories of the Integrated Research Facility at the Rocky Mountain Laboratories, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA.

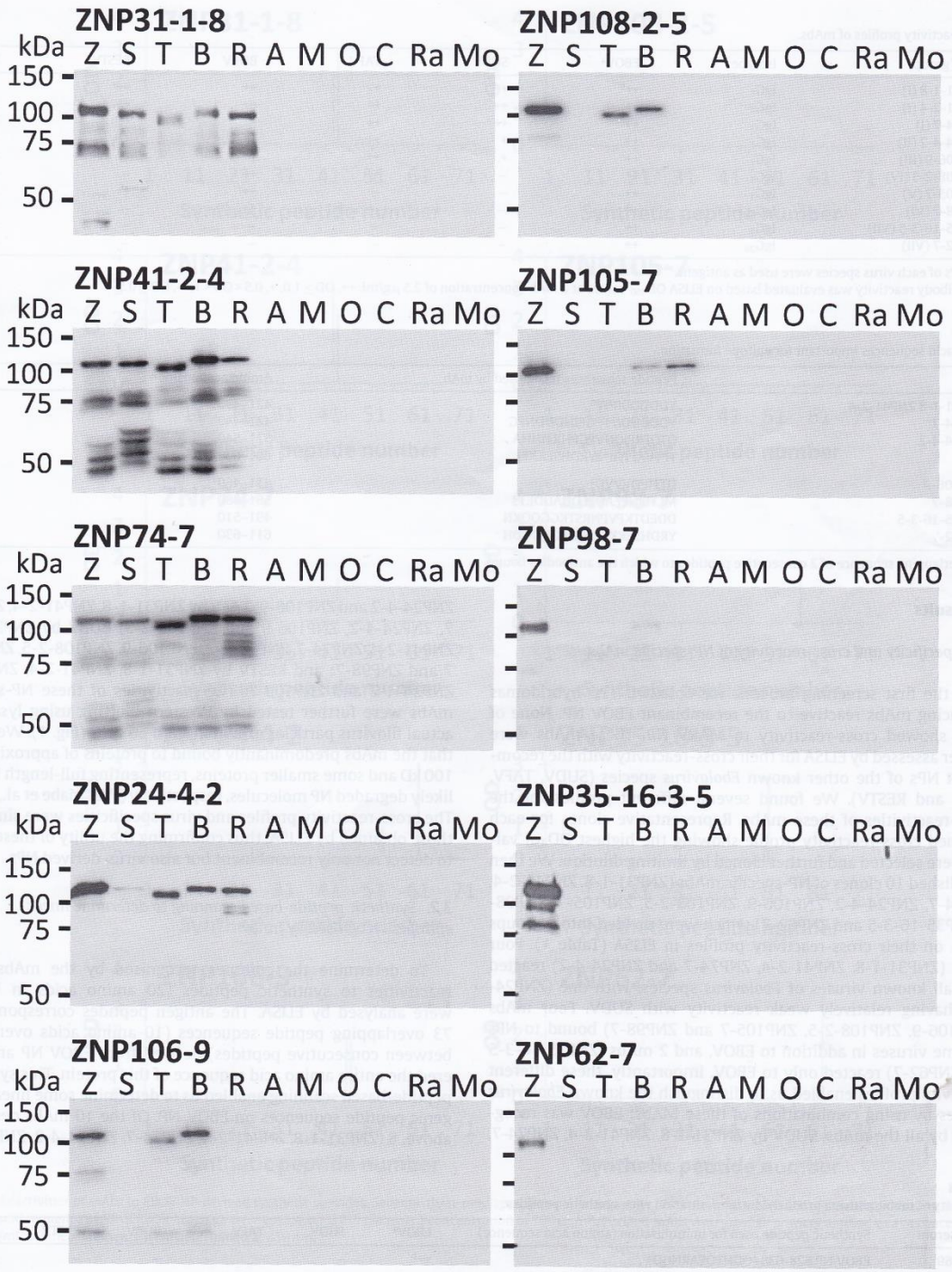

Fig. 1. Reactivity of mouse mAbs in Western blotting. Vero E6 cells were infected with EBOV (Z), SUDV (S), TAFV (T), BDBV (B), RESTV (R), MARV Angola (A), MARV Musoke (M), MARV Ozolin (O), MARV Ci67 (C) or RAVV (Ra). Cell culture supernatants containing virus particles were collected, inactivated and subjected to SDS-PAGE under reducing conditions. Mo, mock-infected.

Table 1
Cross reactivity profiles of mAbs.

mAb (group)	Isotype	EBOV[a]	SUDV	TAFV	BDBV	RESTV	MARV
ZNP31-1-8 (I)	IgG_1	++[b]	++	++	++	++	–
ZNP41-2-4 (I)	IgG_1	++	++	++	++	++	–
ZNP74-7 (I)	IgG_1	++	++	++	++	++	–
ZNP24-4-2 (II)	IgG_1	++	+	++	++	++	–
ZNP106-9 (III)	IgG_1	++	+	++	++	–	–
ZNP108-2-5 (IV)	IgG_1	++	–	++	++	–	–
ZNP105-7 (V)	IgG_1	++	–	–	++	++	–
ZNP98-7 (VI)	IgG_{2a}	++	–	–	++	–	–
ZNP35-16-3-5 (VII)	IgG_1	++	–	–	–	–	–
ZNP62-7 (VII)	IgG_{2b}	++	–	–	–	–	–

[a] VLPs of each virus species were used as antigens.
[b] Antibody reactivity was evaluated based on ELISA OD_{450} values at a mAb concentration of 2.5 μg/ml. ++, OD ≥ 1.0, +, 0.5 < OD < 1; –, OD ≤ 0.5.

Table 2
Amino acid sequences important for epitope formation.

mAb	Peptide sequences recognised by mAb	Amino acid positions
ZNP31-1-8 ZNP41-2-4	YDDDDDIPFP[a]	421–430[a]
ZNP74-7	YDDDDDIPFPGPINDDDNPG	421–440
ZNP24-4-2	QTQFRPIQNVPGPHRTIHHA	521–540
	TPTVAPPAPVYRDHSEKKEL	601–620
ZNP106-9	DTTIPDVVVD[a]	451–460[a]
ZNP98-7	MLTPINEEADPLDDADDETS	561–580
ZNP35-16-3-5	DDEDTKPVPNRSTKGGQQKN	491–510
ZNP62-7	YRDHSEKKELPQDEQQDQDH	611–630

[a] Overlapping sequence of 2 consecutive peptides to which the antibodies bound.

3. Results

3.1. Specificity and cross-reactivity of NP-specific mAbs

In the first screening process, we obtained 126 hybridomas producing mAbs reactive to the recombinant EBOV NP. None of them showed cross-reactivity to MARV NP. These mAbs were further assessed by ELISA for their cross-reactivity with the recombinant NPs of the other known *Ebolavirus* species (SUDV, TAFV, BDBV and RESTV). We found several different profiles for the cross-reactivities of these mAbs. Representative clones for each obtained cross-reactivity profile showing the highest OD_{450} values were selected and further cloned by limiting dilution. We then established 10 clones of NP-specific mAbs (ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2, ZNP106-9, ZNP108-2-5, ZNP105-7, ZNP98-7, ZNP35-16-3-5 and ZNP62-7) which were divided into 7 groups based on their cross-reactivity profiles in ELISA (Table 1). Four mAbs (ZNP31-1-8, ZNP41-2-4, ZNP74-7 and ZNP24-4-2) reacted with all known viruses of *Ebolavirus* species, with one (ZNP24-4-2) having relatively weak reactivity with SUDV. Four mAbs (ZNP106-9, ZNP108-2-5, ZNP105-7 and ZNP98-7) bound to NPs of some viruses in addition to EBOV, and 2 mAbs (ZNP35-16-3-5 and ZNP62-7) reacted only to EBOV. Importantly, these different reactivity profiles enabled us to distinguish the known *Ebolavirus* species by using combinations of these MAbs: EBOV was recognised by all the mAbs, SUDV by ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2 and ZNP106-9; TAFV by ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2, ZNP106-9 and ZNP108-2-5; BDBV by ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2, ZNP106-9, ZNP108-2-5, ZNP105-7 and ZNP98-7; and RESTV by ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2 and ZNP105-7. The reactivities of these NP-specific mAbs were further tested by Western blotting using lysates of actual filovirus particles grown in Vero E6 cells (Fig. 1). We found that the mAbs predominantly bound to proteins of approximately 100 kD and some smaller proteins, representing full-length NP and likely degraded NP molecules, respectively (Watanabe et al., 2006). The cross-reactivity profiles and virus specificities were similar to those obtained by ELISA, thus confirming the utility of these mAbs to detect not only recombinant but also virus-derived NPs.

3.2. Synthetic peptide-based scanning to determine linear epitopes recognised by mAbs

To determine the epitopes recognised by the mAbs, their reactivities to synthetic peptides (20 amino acids in length) were analysed by ELISA. The antigen peptides corresponded to 73 overlapping peptide sequences (10 amino acids overlapped between consecutive peptides) derived from EBOV NP and covered the entire amino acid sequence of this protein. This synthetic peptide-based scanning enabled us to determine some linear antigenic peptide sequences on EBOV NP. Of the 10 mAbs described above, 8 (ZNP31-1-8, ZNP41-2-4, ZNP74-7, ZNP24-4-2, ZNP106-9,

Table 3
Reactivity of rabbit antisera produced by immunisation with synthetic peptides.

Antiserum	Synthetic peptide used for immunisation (amino acid sequence)	EBOV[a]	SUDV	TAFV	BDBV	RESTV	MARV
FS0169	EBOV NP 628-638 (QDHTQEARNQD)	++[b]	–	–	–	–	–
FS0191	SUDV NP 631-644 (QGSESEALPINSKK)	–	++	–	–	–	–
FS0046	TAFV NP 630-643 (NQVSGSENTDNKPH)	–	–	++	–	+	–
FS0048	BDBV NP 628-641 (QSNQTNNEDNVRNN)	–	–	–	++	+	–
FS0170	RESTV NP 630-643 (TSQLNEDPDIGQSK)	–	–	–	–	+	–
FS0610	MARV NP 635-652 (RVVTKKGRTFLYPNDLLQ)	–	–	–	–	–	++

[a] VLPs of each virus species were used as antigens.
[b] Antibody reactivity was evaluated based on ELISA OD_{450} values at a serum dilution of 1:2000. ++, OD ≥ 1.0; +, 0.5 < OD < 1; –, OD ≤ 0.5.

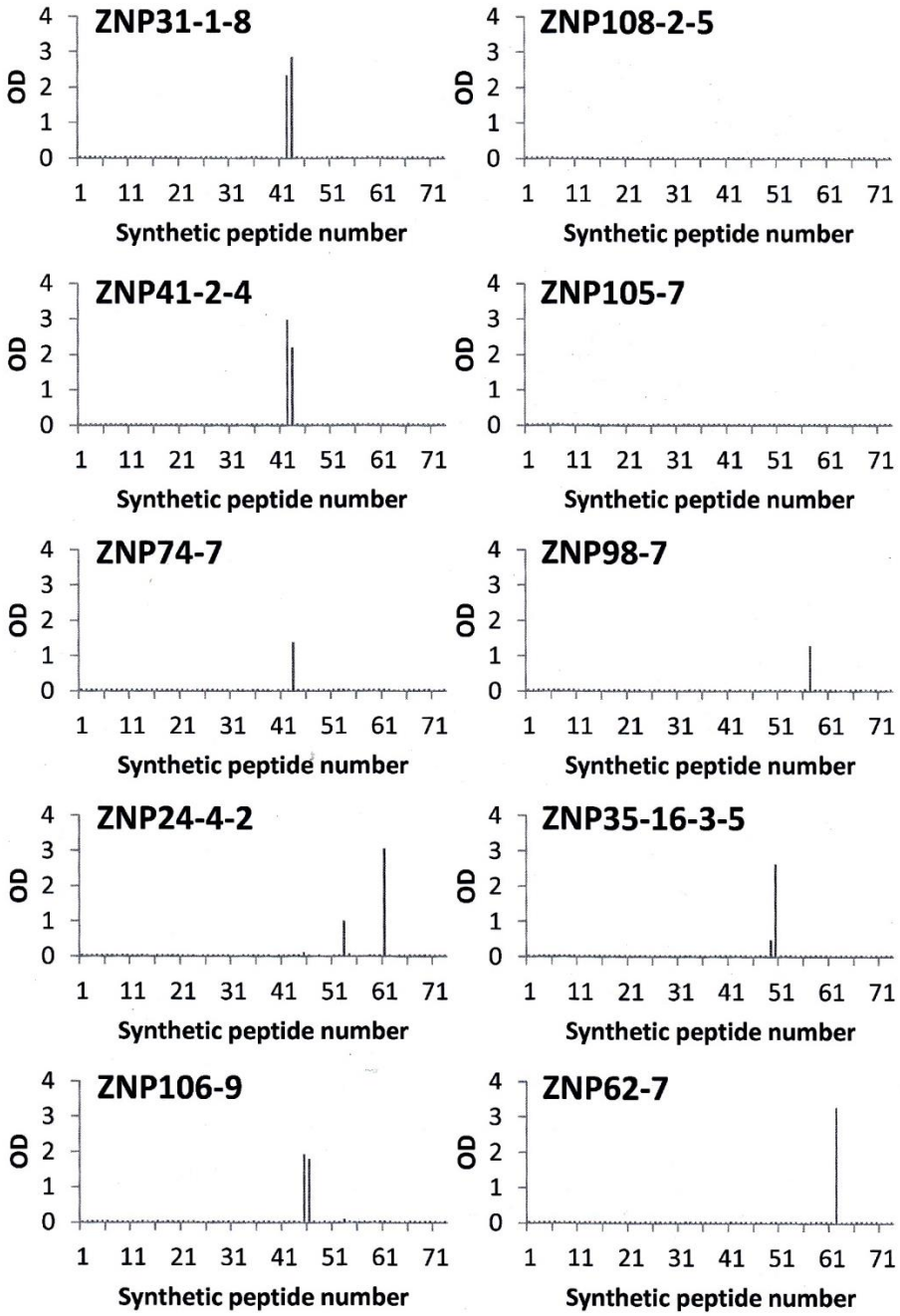

Fig. 2. Reactivities of mAbs to EBOV NP-derived synthetic peptides. Seventy-three overlapping peptide sequences (20 aa in length with a 10 aa overlap) covering the entire amino acid sequence of NP of EBOV Mayinga were coated on ELISA plates at a concentration of 100 μg/ml. Purified mAbs were used as primary antibodies at a concentration of 1 μg/ml. OD measurements were determined at 450 nm.

ZNP98-7, ZNP35-16-3-5 and ZNP62-7) bound to at least one peptide, whereas 2 (ZNP108-2-5 and ZNP105-7) had no positive reaction (Fig. 2). The amino acid sequences recognised by these 8 mAbs are summarised in Table 2 and Fig. 3. Three highly cross-reactive mAbs, ZNP41-2-4, ZNP31-1-8 and ZNP74-7, strongly reacted to the peptide corresponding to aa positions 421–440. ZNP41-2-4 and ZNP31-1-8 reacted further with the consecutive peptides corresponding to aa positions 411–430, restricting the recognised epitope to 10 amino acids (aa positions 421–430). Another cross-reactive mAb, ZNP24-4-2, bound to two peptides

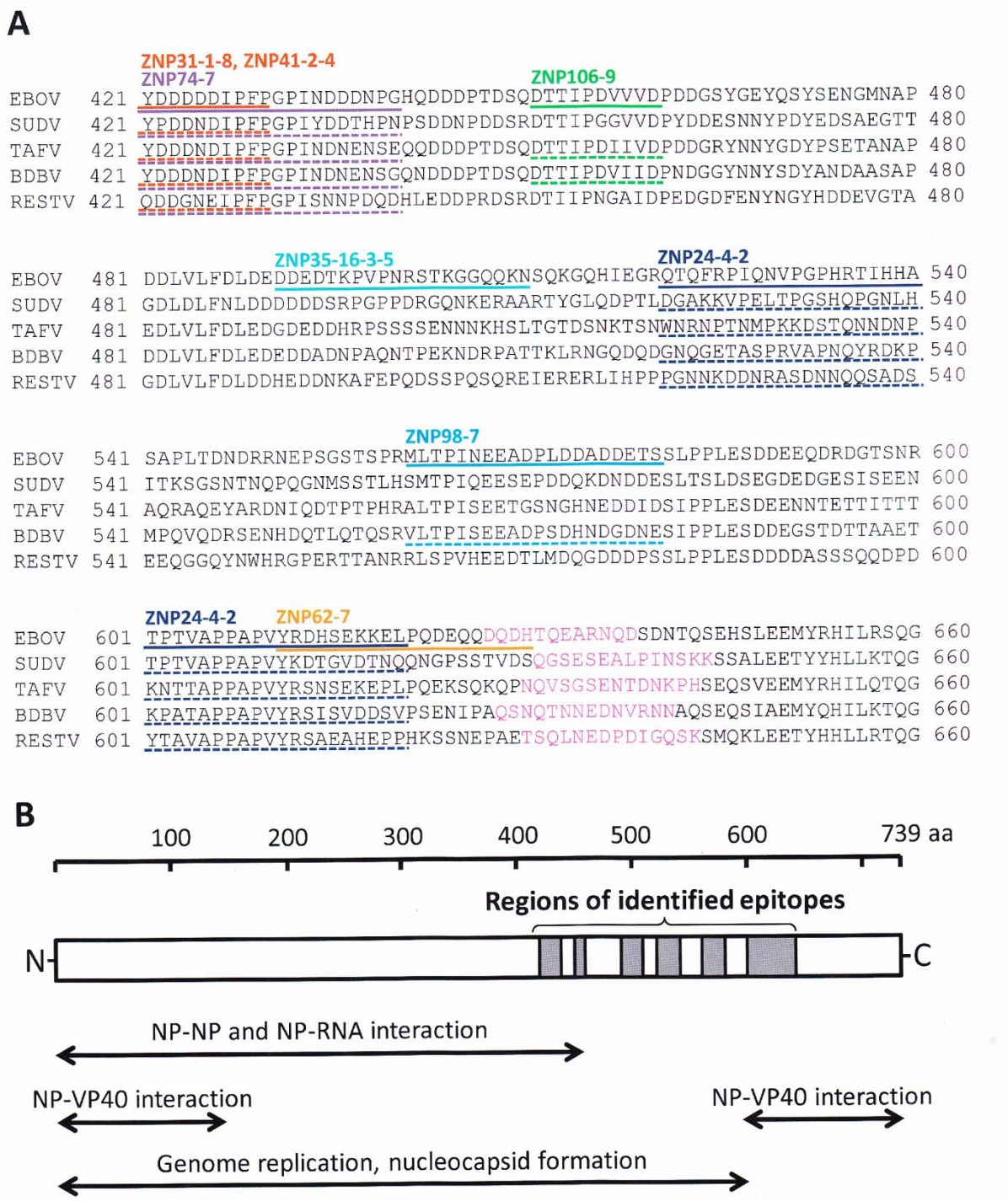

Fig. 3. Epitope sequences in amino acid sequence alignment and known functional regions NPs. (A) Amino acid sequences of EBOV, SUDV, TAFV, BDBV and RESTV were obtained from GenBank under accession numbers AF272001, AF173836, FJ217162, FJ217161 and AF522874, respectively. Amino acid sequences at positions 421–660 of each virus are shown. EBOV NP peptides recognised by the mAbs are highlighted with solid lines. Corresponding regions of the other NPs to which each mAb showed strong cross-reactivity are underlined (dashed lines). Amino acid sequences used for producing species-specific rabbit antisera are shown in pink. (B) Locations of the identified epitopes are shown in the schematic diagram of NP. Functional domains (Bharat et al., 2012; Noda et al., 2007; Watanabe et al., 2006) are also shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

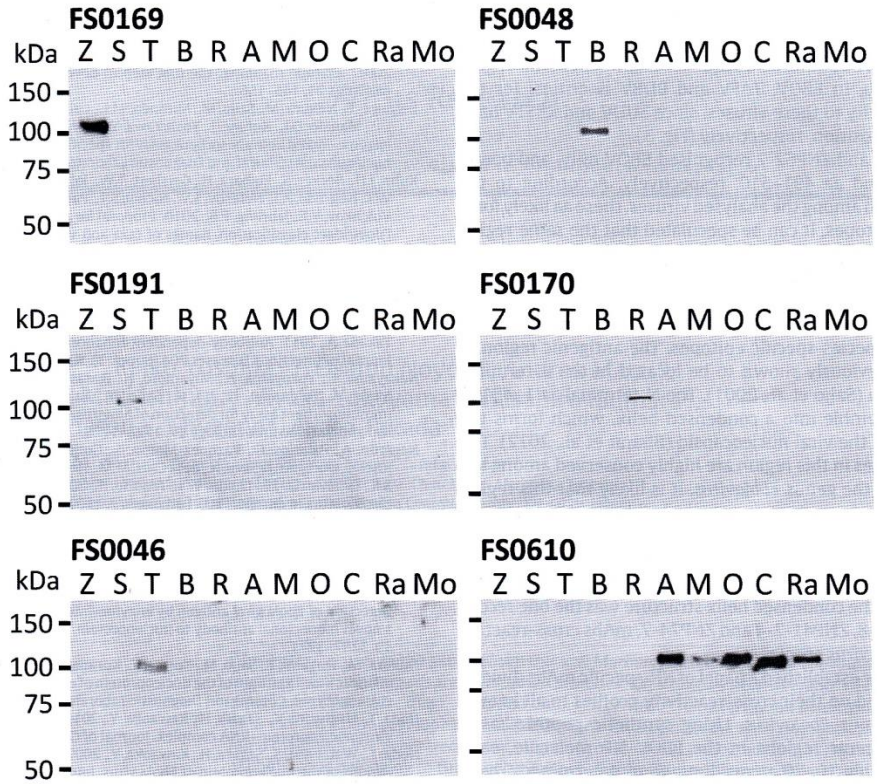

Fig. 4. Reactivity of rabbit antisera in Western blotting. Rabbit antisera (FS0169, FS0191, FS0046, FS0048, FS0170 and FS0610) were produced using synthetic peptides derived from EBOV, SUDV, TAFV, BDBV, RESTV and MARV, respectively. Experimental conditions were the same as in Fig. 1. EBOV (Z), SUDV (S), TAFV (T), BDBV (B), RESTV (R), MARV Angola (A), MARV Musoke (M), MARV Ozolin (O), MARV Ci67 (C), RAVV (Ra). Mo, mock-infected.

corresponding to very different regions in NP. ZNP106-9 reacted with 2 consecutive peptides with overlapping aa sequences corresponding to aa positions 441–460 and 451–470, sharing the 10 aa at positions 451–460. ZNP98-7, ZNP35-16-3-5 and ZNP62-7 each recognised a single peptide derived from different regions of NP (aa 561–580, aa 491–510 and aa 611–630, respectively).

3.3. Reactivity of rabbit antisera produced by immunisation with synthetic peptides

We then sought to determine epitopes distinctive among the NPs of each *Ebolavirus* and *Marburgvirus* species. Based on a programme used to predict B-cell epitopes, we selected region around aa positions 630–650 from viruses representing each filovirus species (Fig. 3) (EBOV, SUDV, TAFV, BDBV, RESTV and MARV), and generated rabbit antisera to the respective synthetic peptides as described in Materials and Methods. The reactivity of each antiserum (FS0169, FS0191, FS0046, FS0048, FS0170 and FS0610) was analysed by ELISA (Table 3). According to the high sequence variation in this region among these viruses, the antisera reacted specifically with the homologous NPs, although FS0046 and FS0048 (antisera to TAFV and BDBV, respectively), showed limited cross-reactivity to RESTV NP. The virus specificity was further confirmed using filovirus lysates in Western blotting (Fig. 4). Notably, all the virus strains tested within the genus *Marburgvirus* (including RAVV) were recognised by antiserum FS0610. These results indicated that the region around aa 630–650 in filovirus NP served as a filovirus species-specific epitope.

4. Discussion

Using mouse mAbs and synthetic peptide-based scanning, we determined 2 highly conserved antigenic regions (aa 421–440 and aa 601–620) serving as linear epitopes in the filovirus NP (Fig. 3). In addition, a stretch of 10 amino acids at aa 421–430 (YDDDDDIPFP) was found to be important for 3 mAbs (ZNP31-1-8, ZNP41-2-4 and ZNP74-7), which strongly recognised all known *Ebolavirus* species. This finding is consistent with a previous study demonstrating that mAbs reactive to EBOV, RESTV and SUDV recognised the sequence at aa 424–430 (Niikura et al., 2003). In this specific region, the amino acid sequence IPFP is completely conserved among all analysed viruses in the *Ebolavirus* genus, suggesting that these aa residues are crucial for conformation of this common epitope.

ZNP24-4-2 was highly cross-reactive to all known viruses of the genus *Ebolavirus*, with weaker reactivity to SUDV (Table 1 and Fig. 1). This mAb reacted with two different peptides corresponding to aa 521–540 and aa 601–620 (Fig. 2). These two peptide sequences may be parts of a conformational epitope. However, there is no conserved sequence in the region at aa 521–540 among all the analysed viruses, whereas the sequence at aa 601–620 shows some conservation. Although SUDV was only weakly recognised by this mAb, this conserved region might be required for recognition as a conserved epitope.

ZNP106-9 and ZNP108-2-5 were strongly reactive to EBOV, TAFV and BDBV, but only weakly reactive or nonreactive to SUDV and RESTV, respectively. This reactivity pattern is consistent with the phylogenetic relationship among the viruses (Towner

et al., 2008). Only ZNP106-9 reacted with the peptide sequence D_{451}TTIPDVVVD$_{460}$, demonstrating that ZNP108-2-5 recognises a different epitope. The amino acid sequence alignment of this region suggests that D_{456} in EBOV, TAFV and BDBV is critical for the ZNP106-9 specificity to these viruses, since SUDV and RESTV have G or N at this aa position, respectively (Fig. 3).

ZNP35-16-3-5 and ZNP62-7 recognised EBOV only, and bound to aa 611–630 and aa 491–510, respectively. According to the sequence variation among the analysed viruses, these aa likely form EBOV-specific epitopes. It can be speculated that the same region of NP of the other viruses in the *Ebolaviruse* genus forms species-specific epitopes. In addition to these two regions, the success of the production of antisera to the synthetic peptides with the predicted sequences around aa 630–650 provided further information on the filovirus species-specific epitopes. The antigenic region of EBOV NP was previously shown to be located in the C-terminal half of the protein (Saijo et al., 2001). The N-terminal aa 1–451 of the EBOV NP assemble into a condensed helix, which forms the inner structure of the viral nucleocapsid (Bharat et al., 2012). The amino acid residues in this region are highly conserved among the known viruses in the genus *Ebolavirus*. It is likely that this region forms functionally important structures inside the NP molecule, and as a result, has limited antigenic properties. This is consistent with our results in which most antigenic regions were found in the highly variable C-terminal region starting at aa 451 (Fig. 3). The only epitope found on the condensed helix structure was the one recognised by ZNP31-1-8, ZNP41-2-4 and ZNP74-7, mAbs cross-reactive to all known *Ebolavirus* species.

In this study, we established a panel of NP-specific mAbs divided into 7 groups based on their cross-reactivity profiles to all known viruses of the genus *Ebolavirus*. Using synthetic peptide-based screening, 8 antigenic regions in the EBOV NP molecule, each consisting of roughly 10–20 aa residues, were determined. These well-characterised mAbs with detailed epitope information should be useful for the development of filovirus antigen detection assays such as immunochromatography-based rapid antigen diagnosis.

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