Neutralising Antibody Activity in Convalescent Plasma of EBOV Survivors

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By

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Abstract

Ebola virus (EBOV), part of the *Filoviridae* family is one of the deadliest pathogens causing highly lethal haemorrhagic fever in humans and non-human primates (NHPs). Since the discovery of the *Ebolavirus* genus, there have been many sporadic outbreaks predominantly in rural settings however, the 2013-2016 EBOV outbreak in West Africa was notable due to widespread virus transmission and was responsible for over 28,000 infected individuals and over 11,000 deaths. The outbreak resulted in the largest population of EBOV survivors to date, revealing many aspects of EBOV infection including sequelae, relapse and providing insights into viral persistence in immune privileged sites and virus transmission via routes with limited previous documentation. Studying EBOV survivors provides a unique opportunity to delineate immune responses controlling viral replication.

The aim of this thesis is to contribute towards understanding the role EBOV specific neutralising Abs (NAbs) present in Ebola survivor convalescent plasma (CP), play in inhibiting EBOV entry into target cells. Specifically, neutralisation was assessed in relation to three different strains of EBOV, to gain a greater understanding of the capacity of CP to neutralise homologous and heterologous EBOV strains. Currently, there is a large population of survivors living in densely urban areas and potentially harbouring the virus in cryptic anatomical sites which indicates that recurrent EBOV outbreaks are highly feasible.

In order to evaluate the neutralisation capacity of CP a pseudo-typed virus (PV) neutralisation system was developed, standardised and utilised in phenotypic assays. PV strains bared the envelope GP of the 2013 epidemic; 1995 Kikwit and a mutant 2013 strain produced to mimic a virus escape mutant. Rigorous control experiments were performed to determine optimal conditions. Comparable results to a reference study for the assessment of EBOV specific antibody (Ab) neutralisation titres was also observed for the PV assay.

The PV assay was used to assess the capacity of CP from a cohort of Ebola survivors to neutralise the EBOV PV variants. A wide-range of neutralisation potential was observed between survivors. To investigate the potential causes, a cross-sectional study was conducted which assessed the IC_{50} titres obtained from CP neutralisation of the EBOV strains with clinical donor parameters. The analysis revealed neither gender, age, BMI, disease

severity nor sampling time could be attributed to the range of EBOV neutralisation observed. Clinical patient parameters have been used to deduce factors associated with EBOV mortality however this is the first documented study assessing clinical parameters in relation to naturally occurring CP NAb responses.

To evaluate CP survivor variations, a longitudinal study was conducted measuring NAb titres from multiple CP donors. CP neutralisation profiles differed between survivors and profiles showing an increasing/ decreasing trend were used to build a mathematical model of CP NAb doubling time and half-life. The persistence and decay of Ab titres are important parameters in assessing efficacy of vaccines. The measurement of CP NAbs with the PV assay was observed to corroborate with an assay measuring total CP anti-EBOV Abs as well as a neutralisation assay using replication competent EBOV (RCE). The assessment of CP neutralisation with RCE is considered the 'gold' standard and highlights the robustness of the developed PV assay. A single donor case study was documented here where a strong boost in NAb titres was measured with the PV, RCE and total anti-EBOV Ab assay. It was also revealed that Abs to EBOV nucleoprotein (NP) and viral matrix protein (VP40) were also boosted, indicating the high probability the donor was exposed to an EBOV antigenic stimulation and indicating the neutralisation immune response was sufficient to control virus activation.

EBOV PV CP neutralisation was measured in the presence of soluble glycoprotein (sGP); the predominant and highly abundant EBOV protein secreted from EBOV infected cells. The observed results indicated that sGP did not affect EBOV infectivity of PV alone; however, in the presence of CP a reversion of PV infectivity was observed in comparison to infectivity of PV incubated with CP alone. This indicates sGP can act as a decoy antigen adsorbing CP NAbs thereby providing for an Ab enhancement to infection (ADE).

This thesis describes the novel study of EBOV specific CP NAbs and the role they have in conferring protection against EBOV. Understanding immune responses associated with survival indicates what an effective vaccine has to confer to protect against EBOV. However, the potential interactions of sGP with CP, highlights an area of research that requires further investigation due to the potential detrimental implications sGP may have in the efficacy of Ab therapeutics.

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Declaration of Authorship

I Charlene Adaken declare that except where indicated by specific reference in the text, this work is my own work. Work done in collaboration with, or with the assistance of others, is indicated as such and acknowledged. The material contained in the thesis has not been presented nor is currently being presented, either wholly or in part, for any other degree or other qualification.

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Charlene Adaken

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List of Abbreviations and Acronyms

ADCC	Antibody-Dependent Cellular Cytotoxicity
ADCP	Antibody-Dependent Cell mediated phagocytosis
A	Alanine
APCs	Antigen-presenting cells
AU	Arbitrary units
BDBV	Bundibugyo virus
BSA	Bovine serum albumin
BSL-2	Biosafety level 2
BSL-4	Biosafety level 4
CAMSs	Cellular adhesion molecules
CatB	Cathepsin B
CatL	Cathepsin L
CCHFV	Crimean- Congo haemorrhagic fever virus
CDC	Centres for Disease Control and Prevention
CFR	Case fatality rates
CHIKV	Chikungunya Virus
CLECs	C-type lectin receptors
CMV	Cytomegalovirus
CNS	Central nervous system
CP	Convalescent plasma
CPE	Cytopathic effect
cRNA	Complementary positive stranded RNA
CSF	Cerebrospinal fluid
Ct	Cycle threshold
DABA	Double Antigen Bridging Assay
DCs	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DEAE	Dextran Diethylaminoethy
DMEM	Dulbecco's modified eagle medium
Dpi	Days post infection
DRC	Democratic Republic of the Congo
EBOV	Ebola Virus
EHF	Ebola haemorrhagic fever
EIAs	Enzyme immunoassays

ELISA	Enzyme Linked Immunosorbent Assay
EM	Electron microscopy
Env	Envelope
ER	Endoplasmic reticulum
EVD	Ebola virus disease
FBS	Foetal bovine serum
FcR	Fc receptor
GFP	Green fluorescent protein
GP	Glycoprotein
GP_1	Glycoprotein 1
GP_2	Glycoprotein 2
H	Hour
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
hMGL	Human macrophage galactose- and N-acetylgalactosamine-specific C-type lectin
HPV	Human Papilloma Virus
HRP	Horseradish peroxidase
HRSV	Human respiratory syncytial virus
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IM	Intramuscular
I	Isoleucine
ITM	Institute of Tropical Medicine
JEV	Japanese encephalitis virus
L	RNA-dependent RNA polymerase
LASV GPC	Lassa virus glycoprotein precursor
LSEC-Tin	Liver and lymph node sinusoidal endothelial cell C-type lectin
L-SIGN	Liver/lymph node-specific ICAM-3 grabbing non-integrin
LSTM	Liverpool School of Tropical Medicine
mAbs	Monoclonal Abs
MERS-CoV	Middle East respiratory syndrome-coronavirus
MHC	Major histocompatibility complex
Min	Minutes
MLB	Mannose-binding lectin
MLV	Murine leukaemia virus
MWCO	Molecular Weight Cut off

MRE	Microbiological Research Establishment
MV	Measles virus H/F
NAbs	Neutralising Abs
NHPs	Nonhuman primates
NIBSC	National Institute of Biological Standards and Control
NiV	Nipah virus
nm	Nanometer
NP	Nucleoprotein
NPC1	Niemann-Pick C1
O/N	Overnight
ORFs	Open reading frames
oSOC	Optimal standards of care
PBS	Phosphate buffered saline
PEI	Polyethylenimine
PFU	Plaque forming units
PHE	Public Health England
PRE	Progesterone response element
PMOs	Phosphorodiamidate morpholino oligomers
PRNT	Plaque neutralisation test
PS	Phosphatidylserine
PtdSerR	Phosphatidylserine receptors
PV	Pseudo-typed virus
RBR	Receptor binding domain
RCE	Replication competent EBOV
RdRp	RNA dependent RNA polymerase
RESTV	Reston virus
RLUs	Relative Light Units
RNA	Ribonucleic Acid
RNP complex	Ribonucleoprotein complex
ROS	Reactive oxygen species
RPM	Revolutions per minute
RRV	Ross River virus
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription polymerase chain reaction
RV GP	Rabies Glycoprotein
S	Seconds
SARS-CoV	Severe acute respiratory syndrome-coronavirus
SFM	Serum free media

SFTSV	Severe fever with thrombocytopenia syndrome virus
sGP	EBOV secreted glycoprotein
siRNAs	Small interfering RNAs
SUDV	Sudan virus
TACE	Tumor necrosis factor-α converting enzyme
TAFV	Taï Forest virus
TAM	Tyrosine family kinase receptors
T	Threonine
TIM	T-cell immunoglobulin and mucin domain family
V	Valine
VP24	Viral protein- minor matrix protein
VP30	Viral protein-replication-transcription protein
VP40	Viral protein- matrix protein
VSV	Vesicular Stomatitis Virus
VSV Vaccine	Vesicular stomatitis virus-based vaccine
WHO	World Health Organisation
WT	Wild-type
YMH	Yambuku Mission Hospital

1 Introduction

1.1 The Discovery of Ebolavirus

In 1976 patients in the Yambuku Mission Hospital (YMH) in northwest Democratic Republic of the Congo (DRC; previously known as Zaire) were presenting with symptoms of a haemorrhagic fever syndrome of an unknown cause (Burke et al., 1978). Around the same time, patients visiting YMH for a variety of conditions including pregnancy were administered vitamins and other medicines by injection; a regular practice preferred by both patients and medical staff. Between the outpatient department, inpatient medicine wards, prenatal and village outreach clinics, five glass syringes and metal needles were repeatedly used with occasional rinsing but no sterilisation. Dozens of patients who had received injections at YMH developed a febrile haemorrhagic fever and died after approximately 1 week as well as many of their close contacts (Burke et al., 1978). Collectively, this indicated that a transmittable agent was responsible for the newly described syndrome.

Later studies revealed that >120 cases had occurred around this period and more than half were associated with injections (Burke et al., 1978). Blood samples were sent to the Institute of Tropical Medicine (ITM; Antwerp, Belgium) and analysed by Guido van der Groen, Rene Delgadillo and Peter Piot. Cytopathic effects were observed when the specimens were inoculated onto Vero cells and electron microscopy (EM) revealed the presence of a Marburg-like virus. The World Health Organisation (WHO) was informed, samples were sent to laboratories with maximum containment for highly pathogenic viruses, including the Microbiological Research Establishment (MRE; Porton Down, UK) as well as the Centres for Disease Control and Prevention (CDC; Atlanta, US) for further research using numerous cell lines and animals models. It was observed that there was no cross-reactivity between serum from a convalescent DRC patient and Marburg virus (MARV) patients when tested against virus isolated from a DRC patient and archived MARV, with a positive result only occurring between DRC sera and DRC virus and likewise MARV sera and MARV (Burke, Declerq and Ghysebrechts, 1978; Breman *et al.*, 2016; Johnson *et al.*, 1977). EM analysis revealed that the new virus comprised filovirus like particle

resembling MARV virus (Figure 1.1). The newly identified virus was named ''Ebola'' after the Ebola river approximately 60 km from Yambuku, to ensure the Yambuku community would not be stigmatised with having such a disease named after it (Henry, 2015).

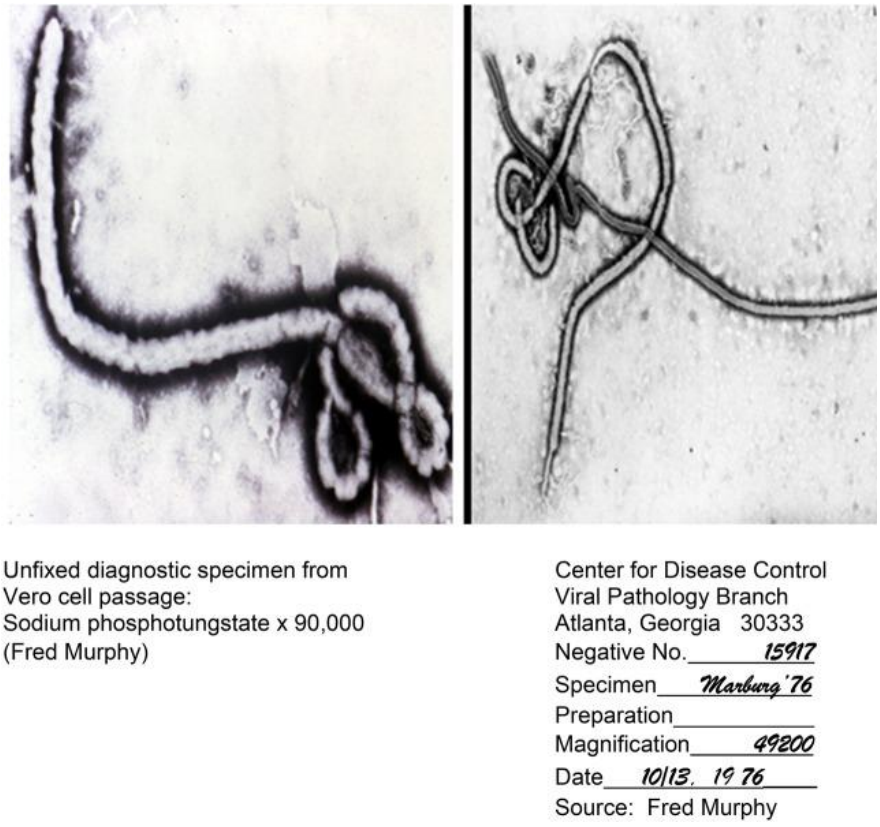


Figure 1.1 An electron micrograph (EM) of the 'unknown' virus alongside Marburg virus (MARV)

The EM pictures of the 'unknown' haemorrhagic fever, subsequently named Ebola virus were taken in October 1976 at the Centers for Disease Control and Prevention (Taken from Breman et al., 2016).

1.2 Taxonomy

The *Ebolavirus* genus is part of the *Filoviridae* family. Since its discovery in Central Africa in 1976, five species falling under the genus have been isolated. The species are named after the region they were discovered, each with one respective virus identified: Taï Forest virus (TAFV), Sudan virus (SUDV), Reston virus (RESTV), Bundibugyo virus (BDBV) and Ebola virus (EBOV) (Kuhn et al., 2010). A summary of the current filovirus taxonomy is shown in (Table 1.1).

Table 1.1 Summary of the filovirus taxonomy

Taken from the ninth International Committee on Taxonomy of Viruses (ICTV) report (Adapted from Kuhn et al., 2014).

Current Taxonomy and Nomenclature
Order *Mononegavirales*
Family *Filoviridae*
Genus *Marburgvirus*
Species *Marburg marburgvirus*
Virus 1: Marburg virus (MARV)
Virus 2: Ravn virus (RAVV)
Genus *Ebolavirus*
Species *Taï Forest ebolavirus*
Virus: Taï Forest virus (TAFV)
Species *Reston ebolavirus*
Virus: Reston virus (RESTV)
Species *Sudan ebolavirus*
Virus: Sudan virus (SUDV)
Species *Zaire ebolavirus*
Virus: Ebola virus (EBOV)
Species *Bundibugyo ebolavirus*
Virus: Bundibugyo virus (BDBV)
Genus *Cuevavirus*
Species *Lloviu cuevavirus*
Virus: Lloviu virus (LLOV)

Within the *Ebolavirus* genus, four species are known to cause Ebola virus disease (EVD) in humans with the fifth only causing disease in non-human primates (NHPs) (Rewar and Mirdha, 2014), of which EBOV is the most characterised. There are a number of EBOV isolates available for use, however, the most utilised for experiments are the “Mayinga” and “Kikwit” strains. The “Mayinga” isolate was the first EBOV isolate obtained (in 1976) and has been widely used for molecular and biological characterisations and the “Kikwit” variant was obtained from the DRC outbreak in 1995 and has been used extensively in NHP studies in the US (Kuhn et al., 2014).

1.3 *Ebolavirus* structure

Filoviruses are negative-sense, single-stranded, ribonucleic acid (RNA) viruses with a membrane envelope. They are filamentous viruses; each filament has a diameter of 80 nm and a length of between 800-1000 nm. Each virion has three compartments; the nucleocapsid, the matrix space and the viral envelope Falasca *et al.*, 2015) (Figure 1.2).

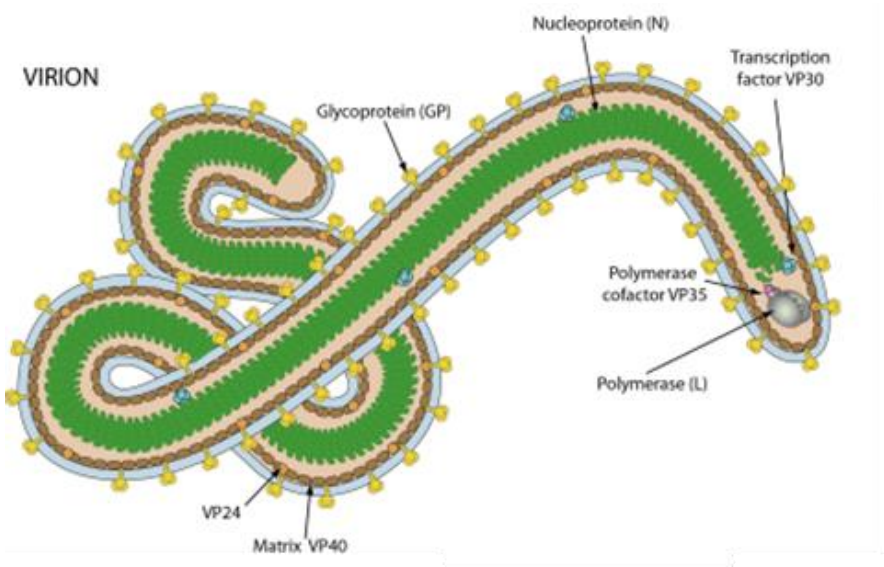


Figure 1.2 The Ebola Virus structure
(Taken from Majid et al., 2016)

The *Ebolavirus* encodes seven structural proteins, nucleoprotein (NP), viral proteins (VP); polymerase co-factor (VP35), matrix protein (VP40), replication-transcription protein (VP30), minor matrix protein (VP24), glycoprotein (GP), and RNA-dependent RNA polymerase (L) (Takada, 2012). The functions of the viral proteins are summarised below (Figure 1.3) (Falasca et al., 2015).

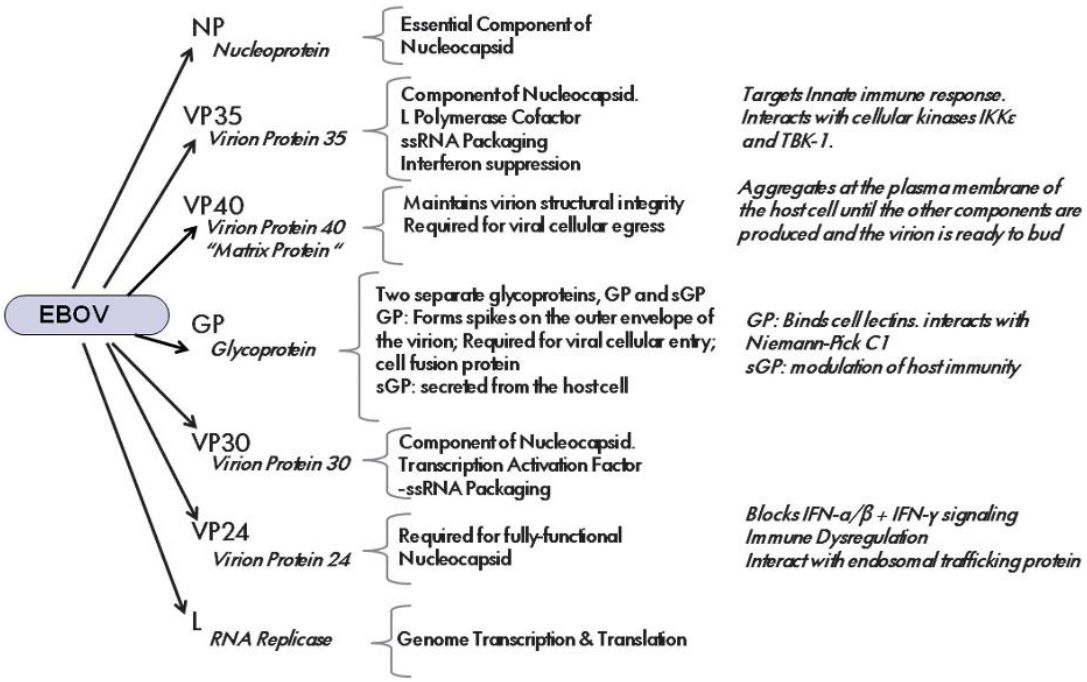


Figure 1.3 Summary of EBOV gene products, function and interaction with host cell

The Ebola virus has seven genes: the NP, the viral proteins VP24-VP30-VP35-VP40, L polymerase, and the GP. The function of the viral proteins in EBOV biology are summarised as well as their interaction with host cells (Taken from Falasca et al., 2015).

1.4 Ebola viral life cycle

1.4.1 Ebola viral entry

Filoviruses have a broad tropism and can infect a variety of different cell types from a wide range of species (Hunt et al., 2012). Unlike other characterised enveloped virus/ cell surface receptor interactions, EBOV GP are not thought to interact with a specific receptor but mediate target cell entry by fairly non-specific receptors. These include C-type lectins (CLECs) and phosphatidylserine (PtdSerR) receptors.

CLECs; LSEC-Tin [liver and lymph node sinusoidal endothelial cell C-type lectin], DC-SIGN [dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin], L-SIGN [liver/lymph node-specific ICAM-3 grabbing non-integrin], mannose-binding lectin [MBL] and hMGL [human macrophage galactose- and N-acetylgalactosamine-specific C-type lectin] interact with N- and O-linked glycans present on the EBOV GP, leading to heightened EBOV entry into target cells (Moller-Tank and Maury, 2015). Studies have demonstrated that the

glycan cap of GP_1 and the mucin domain interact with C-type lectins (Marzi et al., 2006; Takada et al., 2004) however cells lacking CLEC expression are also permissive to EBOV infection indicating their role may be to increase filovirus attachment as opposed to mediating EBOV entry into target cells (Harrison, 2008), which is the function identified in relation to human immunodeficiency virus type 1 (HIV-1) (Figure 1.4) (Matsuno et al., 2010).

PtdSerR functions as an important attachment factor for EBOV GP during entry (Reviewed in Meertens *et al.*, 2012; Moller-Tank *et al.*, 2013; Moller-Tank and Maury, 2014, 2015) which is able to bind to cellular receptors including members of the T-cell immunoglobulin and mucin domain (TIM) family, Tim-1 and Tim-4 and the Tyrosine family kinase receptors (TAM), Tyro3 family (Axl and Mer) (Moller-Tank and Maury, 2015). A study showed that the expression of TIM-1 in cells that were poorly permissive to EBOV, enhanced EBOV entry, whilst loss of TIM-1 surface expression in permissive cells, abrogated filovirus infection (Hunt et al., 2012). It has been demonstrated in Axl-positive cells that infection of replication competent EBOV (RCE) was more efficient than with control cells and EBOV transduction could be blocked by anti-Axl antibodies (Abs) (Shimojima et al., 2006). Increased Axl surface expression was demonstrated to enhance macropinocytosis (Hunt et al., 2011); the principal uptake mechanism for filoviruses (Nanbo et al., 2010), however Axl does not directly interact with EBOV GP and is unlikely to serve as a filoviral receptor (Hunt et al., 2012; Matsuno et al., 2010). Although studies have been carried out to elucidate the interactions between cell surface receptors and EBOV entry, it appears none of these proteins alone are sufficient or necessary for viral entry (Lee and Saphire, 2009). The current lack of clarity regarding which cell proteins acts as specific EBOV receptors suggests that further research including *in vivo* studies are necessary (Moller-Tank and Maury, 2015).

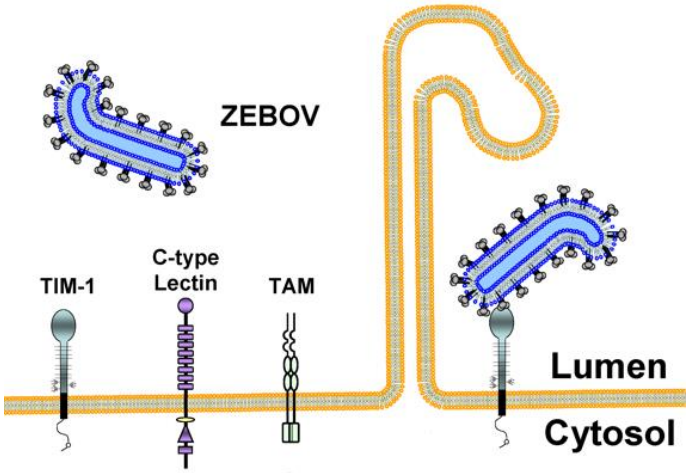


Figure 1.4 Interaction of EBOV with host cell receptors

The glycosylated surface of the virion interacts with attachment factors; C-type lectins and receptors (TIM-1) on the target cell surface, thus concentrating virions on cells prior to internalisation by macropinocytosis. TAM-tyrosine kinase receptors enhance macropinocytosis (Taken from Hunt et al., 2012).

All stages of EBOV entry (binding, internalisation and trafficking to and fusion with the late endosome) are facilitated by the trimeric GP spikes (White and Schornberg, 2012). Although a few routes of EBOV uptake have been reported (Hunt et al., 2012), macropinocytosis is generally accepted as the endocytic pathway for filovirus entry into a target cell (Figure 1.7 Steps 1 +2) (Moller-Tank and Maury, 2015; Nanbo et al., 2010). As described earlier cell surface receptors have been shown to have an effect on EBOV internalisation independent of the viral GP, however the mechanism triggering macropinocytosis remains elusive (Moller-Tank and Maury, 2015).

1.4.2 Trafficking and Priming

Ebola virions are internalised into an early endosomal compartment and subsequently trafficked to the late endosome in a Rab5 and Rab7 GTPase-dependent manner. EBOV GP is categorised as a class I viral fusion protein, these include trimers of a single-chain precursor requiring a proteolytic cleavage (priming) to render it fusogenic (Harrison, 2008); this induces the membrane fusion event resulting in viral infiltration and consequent replication (White and Schornberg, 2012). Full priming of EBOV GP occurs within a target cell when endosomal vesicles mature into the late endosome, the pH drops activating endosomal cysteine cathepsin L (CatL) and B (CatB) which process EBOV GP_1 into smaller proteins (Figure 1.7 Step

3) (Chandran et al., 2005; Schornberg et al., 2006). Priming occurs in two steps, CatL cleaves GP_1 to 20 kDa by, removing the glycan cap and mucin domain, followed by further trimming by CatB, yielding a smaller form of GP_1, between 17-19 kDa (Figure 1.5) (Hood et al., 2010; Schornberg et al., 2006). The two forms of GP_1 have different biochemical and biological properties. Inhibition of CatB activity eliminates EBOV infectivity and also CatB antagonists can strongly inhibit entry of pseudo-typed virus (PV) bearing 20 kDa GP, which is not seen with the 17-19 kDa GP (Schornberg et al., 2006; Wong et al., 2010). Additionally, at high temperatures and low pH the 19 kDa GP binds to liposomes suggesting a fusion-ready form (Brecher et al., 2012; Moller-Tank and Maury, 2015). Although cathepsin cleavage of GP_1 appears essential for viral release into the target cell it has also been described that bacterial thermolysin and proteases present in Vero and mouse embryonic fibroblast cells can efficiently substitute cathepsins (Marzi et al., 2012; Schornberg et al., 2006). However, cleaving of GP is necessary for EBOV release into the target cell and has been proposed to occur in order to expose the receptor binding domain (RBD) of GP to allow interaction with an endosomal receptor (Kaletsky et al., 2007) or potentiate GP for fusion (Schornberg et al., 2006; Wong et al., 2010).

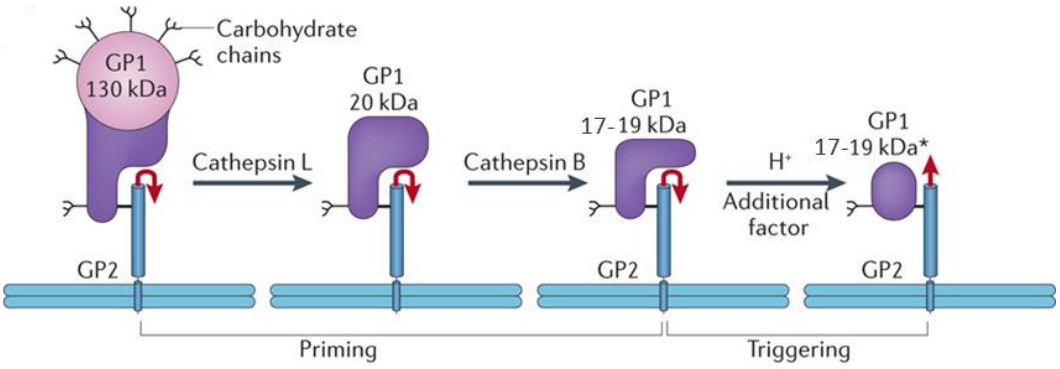


Figure 1.5 Priming of EBOV GP

Cathepsin L (CatL) cleaves the mucin like domain and glycan cap (pink) of the EBOV GP yielding a 20 kDa GP_1, which is further cleaved to 17-19 kDa by Cathepsin B (CatB). Low pH and the activity of a fusion trigger (indicated as an asterix) initiates rearrangement of GP_1, liberating the fusion loop and initiating the fusion cascade (Adapted from White and Schornberg, 2012).

1.4.3 Triggering and Fusion

A fusion trigger i.e. receptor binding and/ or low pH elicits conformational changes promoting fusion. The primed GP_1 has an exposed RBD which binds to the late endosomal protein Niemann-Pick C1 (NPC1). NPC1 is primarily membrane bound and is essential for cholesterol absorption and homeostasis (Hunt et al., 2012). However, this role is independent to its involvement in EBOV entry as cells expressing NPC1 mutants defective in cholesterol egress were still susceptible to EBOV GP mediated infection (Deffieu and Pfeffer, 2011; Lloyd-Evans and Platt, 2010; White and Schornberg, 2012). The interaction with NPC1 is essential for filovirus/ cell membrane fusion, however, whether this is due to the direct interaction of NPC1 with the primed GP_1 (triggering fusion) or whether NPC1 participates (but not solely responsible for fusion) remains unclear (Moller-Tank and Maury, 2015; White and Schornberg, 2012). A hydrophobic fusion loop of GP_2, a protein segment vital for fusion which is normally buried under a GP_1 monomer (Pallesen et al., 2016), becomes exposed by the fusion trigger. The liberated fusion loop is able to bind to the target membrane with the rod shaped intermediate bridging the viral and target membrane and subsequently the fusion protein bends in half pulling the viral and target membrane closer together resulting in the membranes merging (Harrison, 2008; White et al., 2008; White and Schornberg, 2012). The merging of membranes allows the release of the viral RNA and associated proteins into the cytoplasm where viral replication commences (Figure 1.6) (Figure 1.7 Steps 4 + 5) (Hunt et al., 2012).

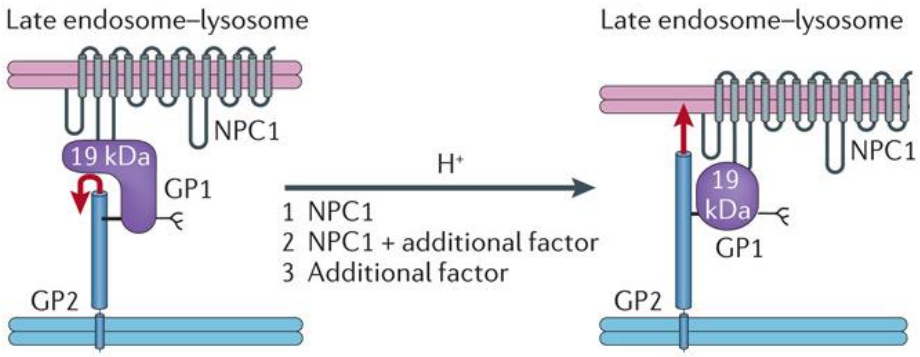


Figure 1.6 Triggering of EBOV GP

The host protein Niemann-Pick C1 (NPC1) either induces or facilitates conformational changes of GP_1, leading to the liberation of a fusion loop in GP_2 (red arrow) which bridges and subsequently permits fusion of the virus and endosomal membranes (Taken from White and Schornberg, 2012).

1.4.4 Transcription and Replication

The EBOV RNA genome is encapsulated by NP and additionally forms a ribonucleoprotein (RNP) complex with RNA dependent RNA polymerase (RdRp) (Bamberg et al., 2005). Post-fusion the RNP is released into the host cell cytoplasm and serves as a template from which complementary positive stranded RNA (cRNA) is produced in the form of RNP and is either further utilised as a template for RNA synthesis or generates viral genomic RNA to be packaged within the virions (Figure 1.7 Step 6 + 7) (Ruigrok et al., 2011; Yu et al., 2017; Zhou et al., 2013).

1.4.5 Assembly and Budding

The endoplasmic reticulum (ER) is the site of synthesis for the GP precursor which is transported via the secretory pathway from the ER via the Golgi apparatus to the plasma membrane (Figure 1.7 Step 8 + 9). The GP precursor undergoes acylation, glycosylation, maturation of N-glycans and lastly proteolytic cleavage with furin (Ito et al., 2001; Ji et al., 2005; Neil et al., 2008). Acylation of viral GP is a post-translational modification involved in virus assembly and budding (Neil et al., 2008; Yu et al., 2017). VP24, a secondary matrix protein contributes to virus assembly (Hoenen et al., 2006; Licata et al., 2004). Silencing of VP24 RNA resulted in the release of reduced virion numbers but did not affect viral transcription and replication, indicating a role in viral assembly and/or budding (Neil et al., 2008), however, the mechanistic details are largely unknown.

It has been demonstrated that NP assembles into helical tubes forming a nucleocapsid-like structure with VP35 and VP24 which accumulates in the perinuclear region (Yu et al., 2017). The complex then migrates via interacting with cellular microtubules facilitated by VP40 to budding sites at the plasma membrane and is incorporated into virions through the NP-VP40 interaction (Baker et al., 2016; Heymann et al., 1980). This mechanism is crucial for nucleocapsid transport to the plasma membrane and incorporation into virions (Yu et al., 2017). VP40 plays a vital role in maintaining structural integrity and maturation of the EBOV virion (Jasenosky et al., 2001; Soni et al., 2013). The interaction of VP40 and the inner leaflet is a major process in budding and is thought to occur via interaction with phosphatidylserine (PS), however, the precise mechanisms are unknown (Jasenosky et al., 2001; Yu et al., 2017). The virions that bud off from the host cell surface are comprised of RNP complex, viral proteins and matrix proteins VP24, VP40 and GP (Figure 1.7 Step 10) (White and Schornberg, 2012).

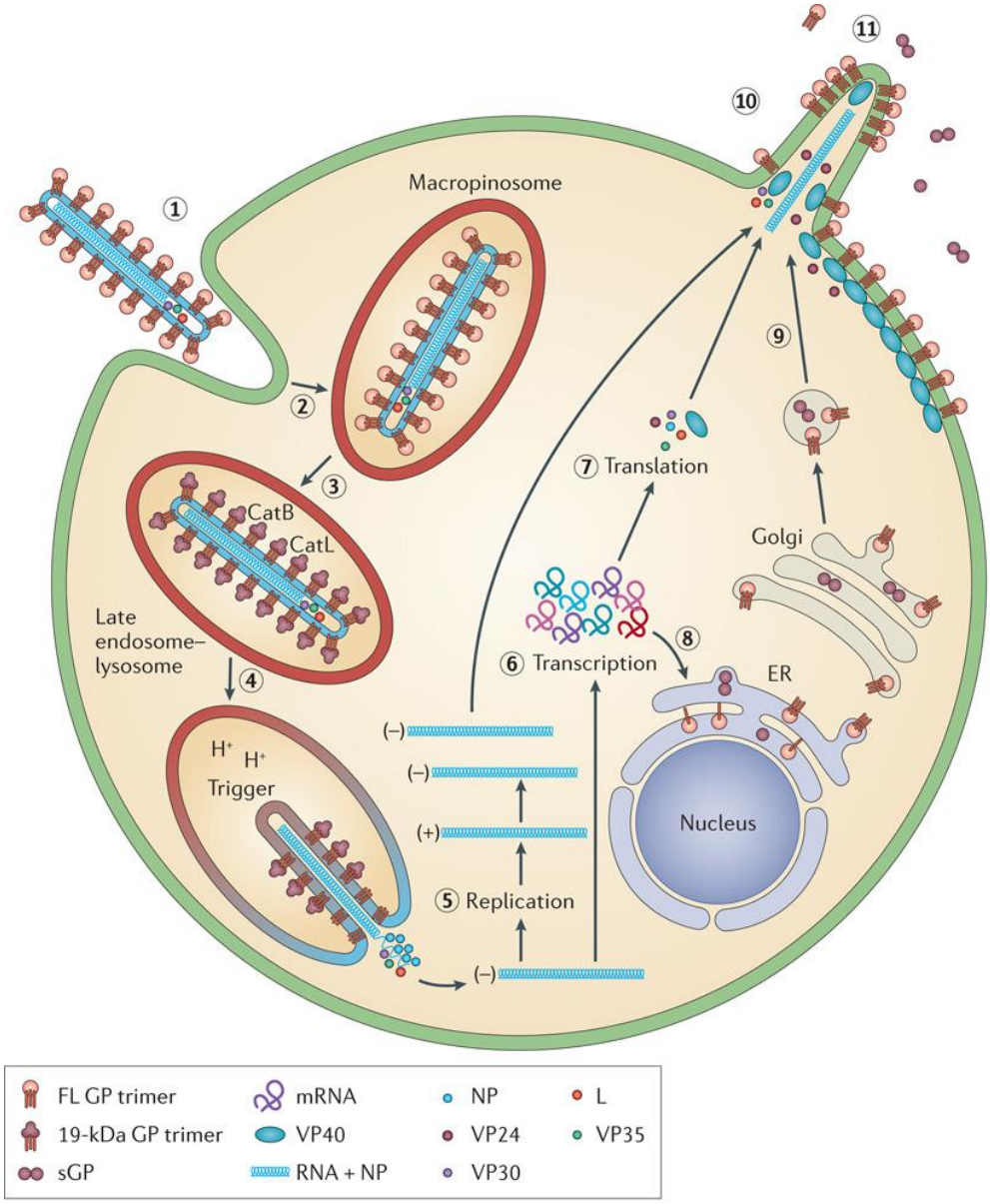


Figure 1.7 The EBOV life cycle

Step 1: EBOV GP binds to host cell attachment factors. Step 2: EBOV is internalised into host cell via macropinocytosis. Step 3: Trafficking to the endosomal compartment occurs where proteases, cathepsin L (CatL) and B (CatB) sequentially cleave the mucin like domain off of GP_1, exposing the receptor binding domain (RBD). Step 4: A fusion trigger initiates viral and endosomal membranes. Step 5: The viral capsid is released from the endosomal compartment, allowing replication of the viral genome. Step 6/ Step 7: EBOV viral proteins, VP35, VP40 and L polymerase aid viral mRNA production which are subsequently translated. Step 8: GP encoded mRNAs are transferred to the endoplasmic reticulum (ER) undergoing synthesis, modification and glycosylation. Step 9: GP are further modified by the Golgi and trafficked to the plasma membrane via vesicles. Step 10: The ribonucleoprotein complex (RNA and NP) with the related viral proteins come together with the matrix proteins; VP24, VP40 and GP to form a virion which buds off the cell surface. Step 11: Secretion of non-structural GP can occur (Taken from White and Schornberg, 2012).

1.5 Epidemiology

As previously described *Ebolavirus* was first documented in 1976 in DRC, concurrently there was an outbreak in Sudan, with case fatality rates (CFR) reaching 52% and 88%, respectively (Feldmann et al., 2002). Outbreaks thereafter continued sporadically and from 1976-2003 10 significant outbreaks occurred with more than 1600 cases of infection and 1100 fatalities recorded. The Reston strain of virus; harmless to humans but lethal for monkeys, was the cause of subclinical infections in the US and the Philippines (Rollin et al., 1999). From 2004-2012 there were 9 documented cases across 4 countries in Africa; Sudan, Congo, DRC and Uganda with a combined death toll of 417 persons (Chippaux, 2014). Limited mention of the outbreaks may have been due to transmission within rural and impoverished settings (Chippaux, 2014).

Between 2013-2016 West Africa witnessed the most wide-spread EBOV outbreak recorded to date (Baize et al., 2014). This epidemic commenced in December 2013, with the index case, a 2-year-old boy from the Gueckedou province in the Republic of Guinea and subsequently the virus, later identified as *Zaire ebolavirus* (WHO, 2014) spread to a separate province 80 km to the east (Carroll et al., 2015). The outbreak was notable due to it being the first geographically dispersed virus transmission in a large, densely populated urban setting as well as taking place in 10 nations that had not previously experienced EBOV (Shultz et al., 2016). The outbreak was considered a pandemic due to the epidemic traversing international borders and affecting several nations. Intense transmission was concentrated to three neighbouring countries; Guinea, Sierra Leone and Liberia, depicted below (Figure 1.8), with cases also reported in Mali, Nigeria, Senegal, Italy, Spain, UK and the US (Shultz et al., 2016). The 2013 outbreak surpassed all preceding outbreaks in total numbers of EVD cases, fatalities, survivors and duration. The epidemic continued for nearly 28 months with the total number of reported cases being 28, 646, deaths of 11, 325 with more than 17, 300 survivors (Shultz et al., 2016). The five subspecies of *Ebolavirus* have a CFR of 25-90% with the Zaire strain being the most fatal, having an overall CFR of 69%-88% (Van Kerkhove et al., 2015). Although the last country to be majorly affected by the 2013-2016 Ebola epidemic was declared “Ebola free” in December 2015, there were still 24 documented ‘flare up’ cases until March 2016. Further cases of Ebola outbreaks in DRC, unrelated to the 2013 epidemic have also been documented to date (November 2018) (Barry et al., 2018; WHO., 2018; WHO., 2018.A). A summary of the outbreaks caused by the *Zaire ebolavirus* to date are summarised (Table 1.2).

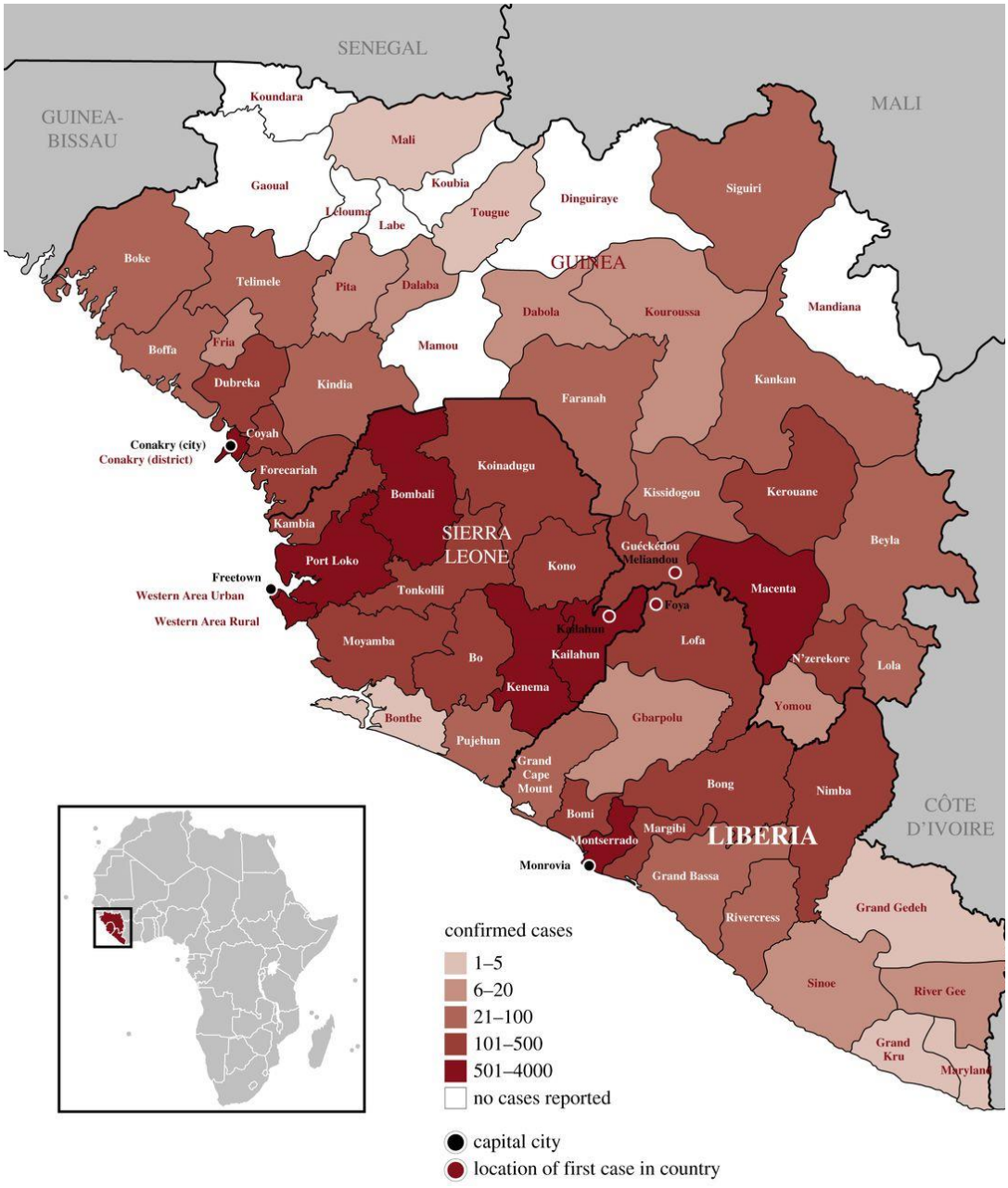


Figure 1.8 Geographical map of Guinea, Sierra Leone and Liberia illustrating total number of confirmed EBOV cases.

(Taken from Coltart et al., 2017 adapted from WHO., 2016)

Table 1.2 All documented Zaire ebolavirus outbreaks to-date (November 2018)

Bold names indicate the initial EBOV occurrence (adapted from Chippaux, 2014)

Year	Country	Districts	Length (weeks)	Number of cases				References
				Presumed	Confirmed	Deaths	Total	
1976	DRC	**Yambuku**, Abumombazi, Kinshasa	9	307	11	280	318	(Pattyn et al., 1977)
1977	DRC	Tandala			1	1	1	(Heymann et al., 1980)
1994	Gabon	**Minkouka**, Andock, Minkébé	13	32	19	31	51	(Amblard et al., 1997; Georges et al., 1999)
1995	DRC	Kikwit, Mosango (±30 villages)	27	233	82	255	315	(Khan et al., 1999; WHO., 1995)
1996	Gabon	Mayibout	12	29	2	21	31	(Georges et al., 1999; WHO., 1996)
1996	Gabon	Booué, Balimba, Lastourville, Libreville	27	56	4	45	60	(Georges et al., 1999; Georges-Courbot et al., 1997)
1996	South Africa	Johannesburg		0	2	2	2	(Milleliri et al., 2004a)
2001-2002	Gabon	Mékambo, Makokou, Franceville	21	37	28	53	65	(Milleliri et al., 2004b; Nkoghe et al., 2005)
2001-2002	Congo	Mbomo, Kellé	20?	50	9	44	59	(WHO., 2002)
2002-2003	Congo	Mbomo, Kellé	17	130	12	128	143	(Formenty et al., 2003)
2003	Congo	Mbomo, Mbandza	7	18	17	29	35	(Boumandouk et al., 2003)
2005	Congo	Etoumbi, Mbomo	6	11	1	10	12	(Nkoghe et al., 2011)
2007	DRC	Luebo	17	<170	>17	186	26	(Clark, Jahrling and Lawler, 2012; Grard et al., 2011)
2008-2009	DRC	Luebo, Mweka	5	<29	>3	15	32	(Grard et al., 2011)
2013-2016	Guinea, Liberia, Sierra Leone, Italy, Mali, Nigeria, Senegal, Spain, US, UK	Geographically wide	112	12, 409	15, 261	11, 325	28, 652	(WHO., 2016.A)
2014	DRC	Boende	12	31	38	49	69	(Maganga et al., 2014)
2017	DRC	Bas Uélé	6	3	5	4	8	(ECDC., 2017)
2018	DRC	Équateur Province	9	16	38	33	54	(WHO., 2018.B)
2018	DRC	North Kivu and Ituri Provinc	13 wks to date- On-going	35	203	155	238	(WHO., 2018.C; WHO., 2018.D)

1.6 EBOV transmission

EBOV is a zoonotic filovirus and outbreaks in human populations have usually required successful cross-species transmission of EBOV from an animal reservoir into humans; specifically to the index case (the primary case), which is known as a 'spillover event' (Chippaux, 2014; Urbanowicz et al., 2016b). This is thought to occur through direct contact with bodily fluids or tissues of EBOV infected animals, their carcasses or waste (Shultz et al., 2016), although the natural reservoir of the virus has not been fully confirmed it is widely considered that bats may be the primary reservoir (Bociaga-Jasik et al., 2014; Urbanowicz et al., 2016b), however, primates including chimpanzees, gorillas and monkeys as well as forest antelopes and porcupines are believed to also harbour the virus (Shultz et al., 2016). Following the 'spillover event', the mode of transmission for secondary cases to arise is from direct human-to-human contact with an infected person, the predominant routes of infection being the mucous membranes, conjunctiva and small breaks in the skin (Peters and LeDuc, 1999; Rewar and Mirdha, 2014). This can occur via the skin or bodily fluids including blood, stool, vomit, urine, faeces, breast milk, semen and potentially saliva and sweat (Bociaga-Jasik et al., 2014; Rewar and Mirdha, 2014) Additionally although the risk is lower, EBOV could be transmitted via fomites e.g. towels, clothing and bedding from the patient (Chippaux, 2014). Aerosol dissemination of EBOV has also been debated however there is little support for this (Osterholm et al., 2015). Intimate exposure with an infected persons can arise when there is an obligation to care for sick family members as well as performing traditional cultural rites at funerals e.g. touching the bodies of those who have died of Ebola virus disease (EVD) (Nkangu et al., 2017).

1.7 EBOV persistence

Scarce data from previous outbreaks suggested there was an extended presence of EBOV nucleic acids in bodily fluids collected from convalescent patients (Bausch et al., 2007; Rodriguez et al., 1999; Rowe et al., 1999; Whitmer et al., 2018). The large population of EVD survivors from the recent epidemic allowed persistence studies to be undertaken, revealing sequelae, the possibility of relapse including detailed insights into viral transmission via routes with limited prior documentation (Chughtai et al., 2016) as well as viral persistence in immune privileged sites where EBOV was detected in biological compartments but no longer in the blood. EBOV has been detected in vaginal secretions, rectal swabs (Rodriguez et al.,

1999), the central nervous system (CNS) (Jacobs et al., 2016), ocular fluid (Steptoe et al., 2017; Varkey et al., 2015), stool (Mora-Rillo et al., 2015), urine, sweat (Kreuels et al., 2014), saliva, tears (Bausch et al., 2007), amniotic fluid, placenta (Caluwaerts et al., 2016), breast milk (Moreau et al., 2015) and semen (Deen et al., 2015). A case report also documented the late viral relapse of a survivor who developed progressive meningoencephalitis with EBOV detected in the CSF (Jacobs et al., 2016).

EBOV persistence in breast milk and seminal fluid likely pose the greatest threat of transmission as has been documented in case studies (Diallo et al., 2016; Mate et al., 2015; Sissoko et al., 2017b). It has been suggested that EBOV infects breast milk at a later stage of disease and is able to be transmitted from both symptomatic and asymptomatic mothers to the child (Nordenstedt et al., 2016; Sissoko et al., 2017b). Regarding semen of EVD survivors, EBOV RNA could be detected up to ~2 years after recovery and from a subset of these specimens, live virus could be isolated (Barnes et al., 2017; Deen et al., 2015; Sissoko et al., 2017a; Uyeki et al., 2016) It was also shown that a new cluster of EVD cases in Guinea and Liberia in 2016 was the result of sexual transmission of EBOV from a survivor who showed EBOV in seminal fluid 531 days after disease onset (Diallo et al., 2016). Based on these results WHO revised their recommendations advising male survivors to refrain or use barrier protection during sexual intercourse for 3 months after recovery (WHO., 2014), to include periodic EBOV RT-PCR semen testing or continue using protection during sexual intercourse for at least 12 months post symptom onset (Whitmer et al., 2018; WHO, 2016.B). The large population of survivors and especially many showing post-EVD sequelae indicates the conceivable re-emergence of EBOV (Vetter et al., 2016).

1.8 EBOV pathogenesis

1.8.1 Clinical Symptoms

Pathogenesis is the process by which EBOV infection leads to EVD. High-level EBOV replication at the portal of entry is associated with dissemination of the virus to multiple cell types which can cause the different immune responses to both become suppressed and over activated, as well as causing disordered coagulation and tissue damage due to a direct viral or indirect host-mediated effector mechanisms (Baseler et al., 2017; Marcinkiewicz et al., 2014).

The onset of EBOV–induced disease is sudden with an incubation period of 2-21 days. Initial clinical symptoms may present as flu-like symptoms; fever, chills, myalgia, malaise and headaches. As the disease progresses a multisystem involvement is demonstrated with systemic, gastrointestinal, respiratory, vascular and neurological symptoms manifesting in patients. Symptoms exhibited may include maculopapular rash, petechiae, with terminal stages of disease characterised by vascular permeability, coagulation disorders, haemorrhaging and death usually as the result of multi-organ failure and a decrease in plasma (Figure 1.9) (Sullivan et al., 2003; Wong et al., 2014).

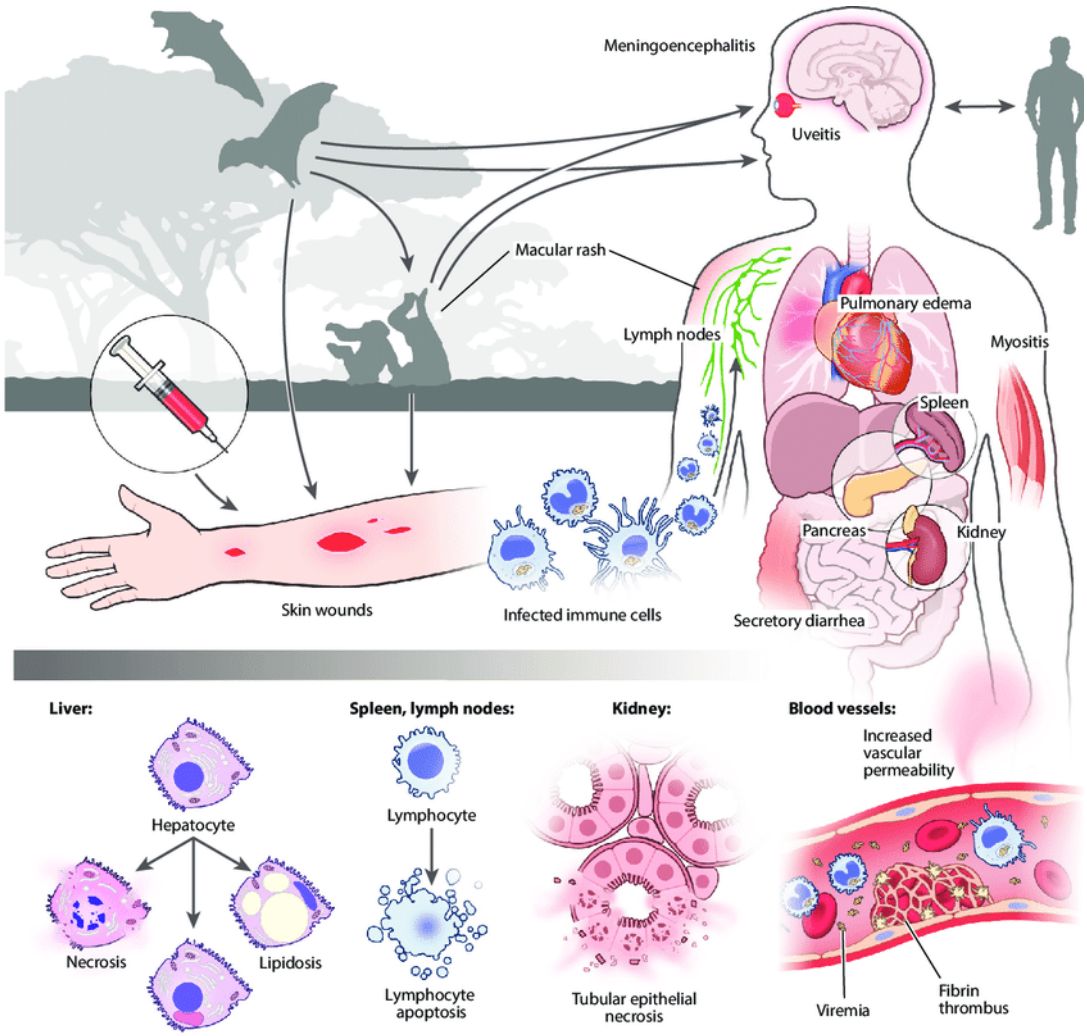


Figure 1.9 EBOV transmission and pathogenesis

EBOV transmission via zoonosis, nosocomial, or person-to-person leads to viral infection of mononuclear phagocytes, which transports the virus to regional lymph nodes. Replication is followed by viral dissemination, leading to tissue and vascular damage (Taken from Baseler *et al*.,2017).

1.8.2 Immune Dysregulation

Global immunosuppression has been suggested as a strategic pathogenic mechanism of EBOV (Mandl and Feinberg, 2015). At the cellular level, EBOV has a broad tropism consisting of sentinel immune cells and antigen-presenting cells (APCs), including macrophages and dendritic cells (DCs) at the initial site of virus infection and replication. *In vitro* interactions between cells in culture and EBOV have shown three EBOV viral proteins; VP24, VP35 and GP have an impact on cell-intrinsic resistance pathways causing inhibition of type I interferon (IFN) production, weakening the effect of IFN on infected cells and thwarting the effects of tetherin, a host factor preventing release of virions (Mandl and Feinberg, 2015; Misasi and Sullivan, 2014).

Widespread Infection initially causes uncontrolled release of pro-inflammatory cytokines and chemokines including IL-1, IL-6, IL-8, TNF-α, monocyte chemotactic protein-1, eotaxins (eosinophil chemoattractant) as well as free radicals. This cytokine storm attracts inflammatory cells; neutrophils and eosinophils to the site of infection in a positive feedback loop, inducing coagulopathy and increased vascular permeability (Marcinkiewicz et al., 2014; Wong et al., 2014) which results in significant damage to tissues and organs (Younan et al., 2018). Reactive oxygen species (ROS) are extremely toxic to lymphocytes (Hildeman et al., 2003) and where levels were found to be significantly higher in fatal human EBOV cases than survivors of EVD (Younan et al., 2018). Additionally, infected DCs do not secrete IFN-α, undergo activation or maturation and do not up regulate major histocompatibility complex (MHC) molecules which would otherwise stimulate T cells (Bosio et al., 2003; Mahanty et al., 2003) which, subsequently impairs the adaptive immune system. T cells that do migrate to this environment with heightened inflammatory mediators, consequently end in bystander-mediated cellular death due to prolonged exposure being toxic (Younan et al., 2018).

It is hypothesised that the lymphatic system is used extensively as the mode of transport for the spread of EBOV (Hensley et al., 2002) to endothelial cells, secondary lymphoid organs and several cell types found in the liver, spleen and lung for further replication (Marcinkiewicz et al., 2014; Wong et al., 2014). Uncontrolled viremia after infection is attributed to most of the observed damages. Together with dysregulated inflammatory responses and a privation of adaptive immunity the analysis of human tissue has shown a correlation between tissue damage and viral antigen levels and nucleic acids, all indicating

damage from viral replication can impact multi-organ failure (Figure 1.10) (Wong et al., 2014).

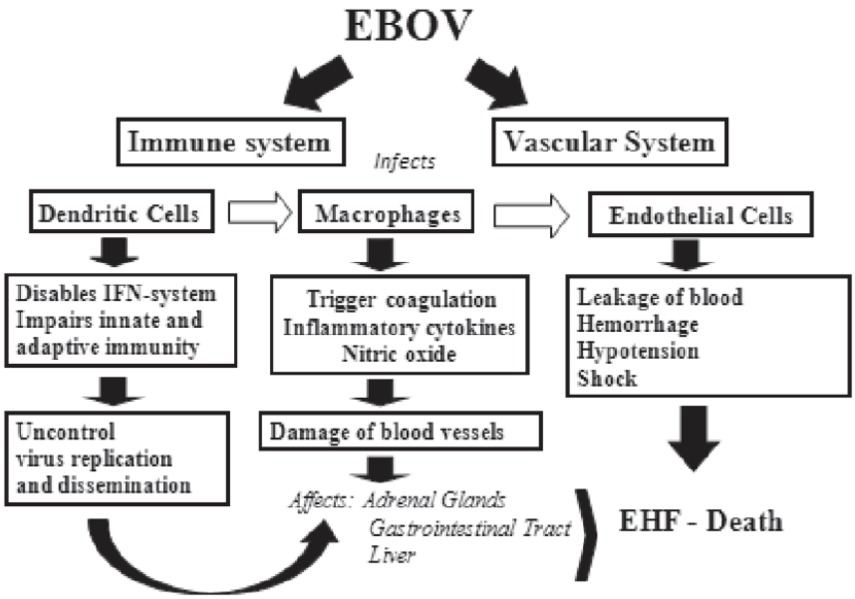


Figure 1.10 Pathological events associated with EBOV infection and Ebola haemorrhagic fever (EHF)

(Taken from Marcinkiewicz et al., 2014)

1.8.3 Indicators of long term immunity

For humans to survive EBOV infection, a robust, specifically adaptive immune response is required (Wong et al., 2014). Immune responses compared between survivors and non-survivors during two 1996 outbreaks in Gabon found that heightened IgM and IgG Ab responses, followed by activation of cytotoxic T cells was related to survival and likewise impaired Ab responses was associated with increased fatality (Baize et al., 1999). A follow up study showed asymptomatic contacts of symptomatic patients developed IgM responses 10-18 days after assumed exposure and IgG, ~1 week after the appearance of IgM. Characterisation of these immune responses revealed T cell response was likely responsible for the removal of infected cells and for the generation of EBOV-specific IgG1 and IgG3 subclasses, which were thought to limit the spread of EBOV to immune privileged sites (Leroy et al., 2001; Wong et al., 2014). To characterise sustained immune responses, NHPs were administered a combination of three EBOV GP specific monoclonal Abs (ZMapp™) resulting

in protection following EBOV challenge. EBOV re-infection revealed GP-specific IgG Ab levels correlated with survival, as non-surviving NHPs demonstrated a 10-fold decrease in IgG titre measured in serum harvested at the time of re-challenge. NHPs that succumbed with re-infection were concluded to have developed a non-protective memory response post initial infection (Qiu et al., 2013; Wong et al., 2014).

Specific IgG Abs were detected in some samples of survivors years after various outbreaks, including: 14 survivors, 2 years after the 1995 Kikwit outbreak (Ksiazek et al., 1999), 2 survivors, 10 years after and 14 survivors, 40 years after the 1976 Yambuku outbreak (Misasi and Sullivan, 2014; Rimoin et al., 2018). Some survivors from the outbreaks that occurred between 1996 and 2001 in Gabon showed detectable IgG levels in 2007, however, concurrently there was a decline in detectable IgG levels in other survivors (Wauquier et al., 2009; Wong et al., 2014). Although IgG levels may be an indicator of long term protection in EBOV survivors, further research is needed in order to specify immunologic correlates that can predict protective immunity (Mandl and Feinberg, 2015).

1.9 Ebola Glycoproteins (GP)

The EBOV envelope is composed of multimers of a single structural GP embedded into a lipid membrane. Co-transcriptional editing and post-translational processing of the GP gene and gene products results in more than three variant GP forms being produced (Lee and Saphire, 2009; Sanchez et al., 1996). The EBOV GP gene encodes the non-structural secreted glycoprotein (sGP), the transmembrane glycoprotein ($GP_{1,2}$), as well as a small soluble glycoprotein (ssGP) (Mehedi et al., 2011).

1.9.1 GP gene products

1.9.1.1 sGP

The gene arrangement of filoviruses are linear and the GP gene is located fourth from the 3' end (Sanchez et al., 1996). All EBOV GP genes have a well conserved open reading frame. Seven consecutive adenosines are encoded by the GP gene within a predicted hairpin loop. The primary product produced from transcription of the GP gene is a 364 residue, non-structural sGP (Lee and Saphire, 2009). sGP is first synthesised as pre-sGP, a golgi specific precursor from which the mature form of the protein is generated following post-translational proteolytic cleavage at its C-terminus by furin, a cellular protease (de La Vega,

Wong, Kobinger, *et al.*, 2015). All five species of EBOV encode sGP by the GP gene (de La Vega et al., 2015). As well as sGP, cleavage at the C-terminus yields Δ-peptide, a small non-structural secreted protein which undergoes multiple post-translational modifications such as heavy O-linked glycosylation (de La Vega et al., 2015; Lee and Saphire, 2009). A homodimer of 100 kDa is formed by monomers of sGP binding to each other in a parallel orientation through di-sulphide bonds at residues Cys53 and Cys306. In addition, sGP has six predicted N-linked glycosylation sites and sGP is efficiently secreted from infected cells ((Figure 1.11 (1) + Figure 1.12) (Volchkova et al., 1998).

1.9.1.2 GP

The virion surface (spike) GP is produced as a result of RNA editing of the GP gene which occurs in approximately 25% of transcripts (Cook and Lee, 2013). The L polymerase stutters at the site of the seven adenosines within the hairpin loop, resulting in the insertion of an additional adenosine and a frame shift in the coding RNA. This allows for the fusion of two separate open reading frames (ORFs) and results in the production of the surface glycoprotein $GP_{1,2}$ (de La Vega, Wong, Kobinger, *et al.*, 2015), a type I transmembrane protein which is 676 residues long and shares 295 amino acids at its N-terminus with sGP. The difference in C-termini leads to variant structures and functions of the proteins. EBOV pre-GP is cleaved by furin to form the two subunits GP_1 and GP_2 which remain linked to each other by a di-sulphide bond. The heterodimer (GP_1 and GP_2) assembles into a 450 kDa trimer at the surface of the virion (Lee and Saphire, 2009). N and O–linked glycans encompass the surface of the GP molecule. EBOV GP also has a mucin-like domain that is heavily modified with O-linked glycans (Cook and Lee, 2013). The tumour necrosis factor-α converting enzyme (TACE), can cleave and release the trimeric GP releasing it from the membrane, which is termed shed GP (Figure 1.11 (2) + Figure 1.12) (Cook and Lee, 2013).

1.9.1.3 ssGP

Whilst stuttering of the L polymerase can lead to an insertion of an adenosine as described above, it can also result in the deletion of one, or addition of two adenosines leading to the production of a third protein ssGPP. ssGP is a small, non-structural sGP which also shares 295 N-terminal residues with sGP and GP but differs in its C-termini (Figure 1.11 (3)) (Lee and Saphire, 2009). ssGP is thought to be glycosylated with N-linked carbohydrates (Mehedi et al., 2011) which have been documented in promoting expression, cellular attachment, protection from proteases and Ab evasion (Lennemann et al., 2015).

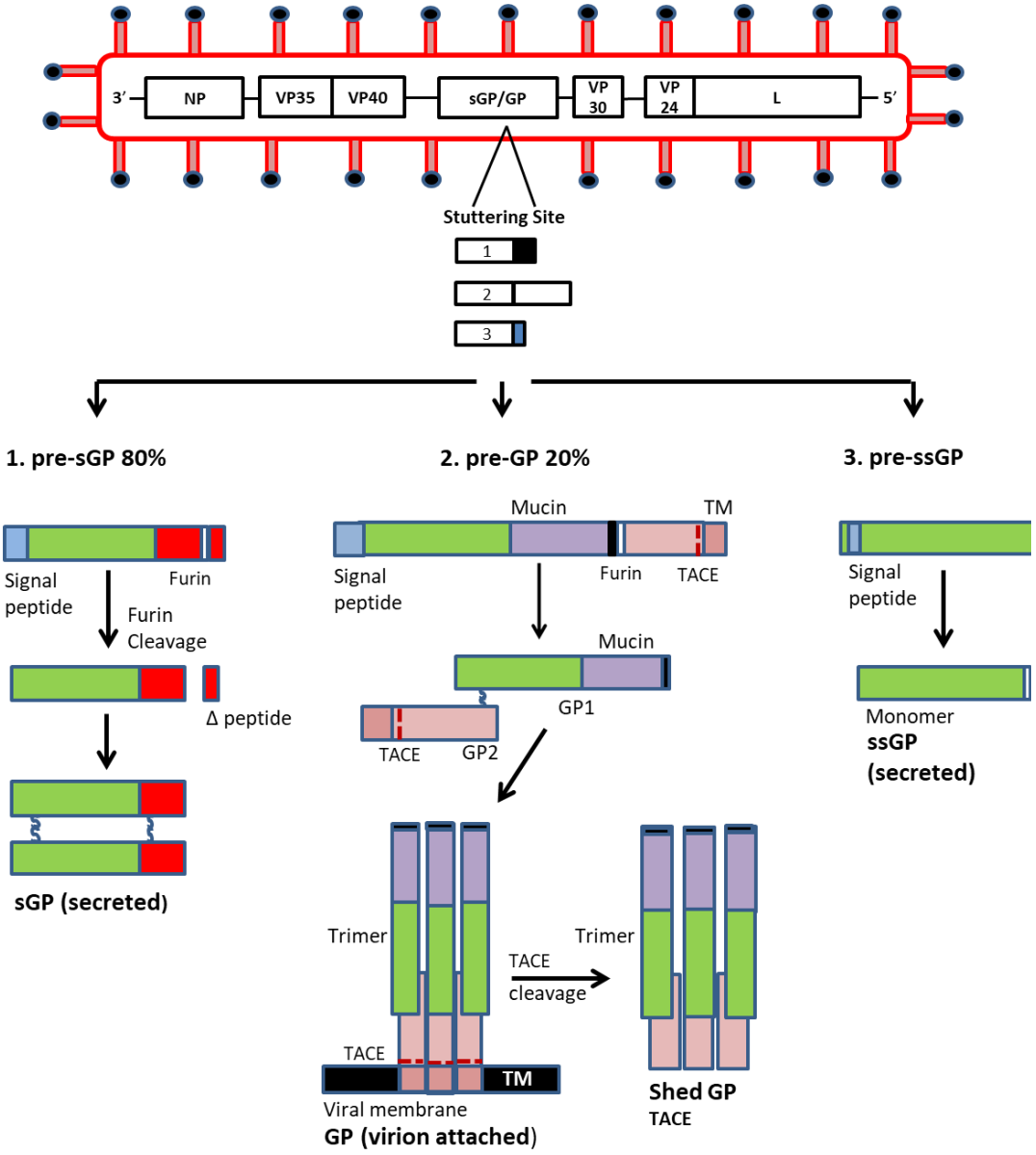


Figure 1.11 Arrangement of EBOV genes and GP produced as a result from RNA editing

sGP is the primary GP molecule produced from 80% of transcripts, it is cleaved at the C-termini to produce Δ-peptide and is secreted as a homodimer from infected cells. The spike GP is produced from 20% of transcripts and forms a heterodimer transmembrane protein which can be cleaved by TACE to release the GP. A minute number of transcripts encode a homodimer, ssGP which can also be secreted from infected cells (Adapted from Cook and Lee, 2013).

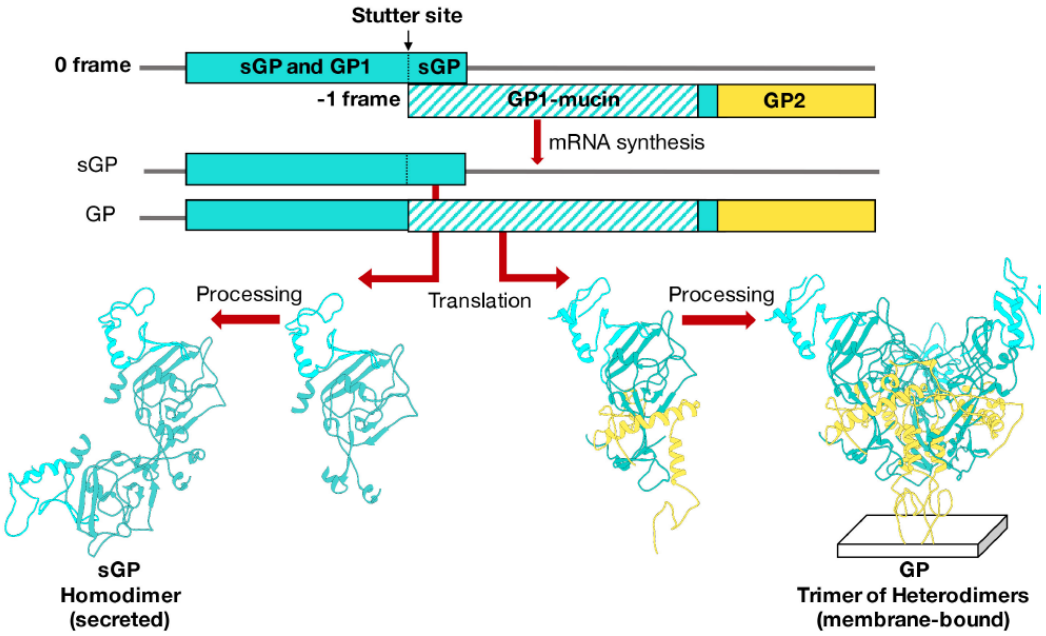


Figure 1.12 sGP and GP gene comparison, mRNA, translation, processing and oligomerisation

The GP gene encodes for both sGP and GP. sGP mRNA is produced from a read-through of GP in the zero frame by L-polymerase with the remaining GP portion in this frame an extended non-coded region. The L-polymerase stutter at the 7-adenosine patch causing a shift into the +1 reading frame, resulting in GP mRNA, including the mucin like domains and GP_2 containing the transmembrane region. Glycosylation of both proteins occurs in the Golgi, sGP is secreted as a soluble dimer into the serum whilst GP becomes anchored to the cell-membrane as a trimer of heterodimers. The glycan cap shared between GP and sGP is shown in cyan and the core largely similar between the two proteins is shown in sea green. GP_2 is shown in yellow (Taken from Pallesen *et al.*, 2016).

1.9.2 Role of the GP gene products

Although not completely defined it has been suggested that the viral GP is crucial in the manifestation of EBOV infection (Sullivan et al., 2003).

1.9.2.1 sGP

sGP has been detected in the blood of acutely infected patients at high concentrations (Sanchez and Ksiazek, 1999) as well as NHPs, although its biological significance has remained elusive (Takada and Kawaoka, 2001). The importance of RNA editing to regulate sGP and $GP_{1,2}$ expression was highlighted in a study which produced a recombinant EBOV with a deletion of the editing site which was unable to produce sGP. *In vitro* infections with the

recombinant virus compared to wild-type (WT) saw a significant increase in cytopathogenicity, however, *in vivo* studies revealed GP production alone was associated with reduced pathogenicity which ultimately reverted to a WT editing site with normal sGP expression levels and a highly pathogenic phenotype (Yang et al., 1998). This favoured expression profile suggests an importance for sGP for virus survival within the host (de La Vega, Wong, Kobinger, *et al.*, 2015). Various ideas have been proposed for the roles of sGP secreted from infected cells in relation to EBOV pathogenesis and interference with host cell immunity.

1.9.2.1.1 sGP the decoy antigen

One theory is that sGP can serve as a decoy antigen for EBOV-specific immune responses and therefore aide in EBOV pathogenesis. sGP has been found to cross-react with Abs present in the sera of human EHF survivors and (Maruyama et al., 1999a) likewise neutralise the activities of GP specific anti-sera (Ito et al., 2001). It was hypothesised that sGP could have an effect on the host immune responses by adsorbing stimulated Abs raised against $GP_{1,2}$ as both sGP and $GP_{1,2}$ share a common N-termini region (Figure 1.13 C).

1.9.2.1.2 sGP antigenic subversion

It has been demonstrated that sGP can compete for binding to anti-$GP_{1,2}$ Abs that were induced by sGP but not by $GP_{1,2}$ (Mohan et al., 2012). It was also shown that if animals were pre-exposed to sGP, the molecule was able to impede Ab-mediated neutralisation against the virus (Mohan et al., 2012). The proposed model considered that a repertoire of naïve B cells recognise both $GP_{1,2}$ and sGP, however, as sGP is produced more abundantly specifically; ~35 sGP dimers and 8 GP trimers for every 100 monomers produced from the GP gene, B cell recognising epitopes shared between the two isoforms would more likely encounter sGP. It was hypothesised sGP could cause the host immune response to be redirected to inaccessible, non-existent epitopes in $GP_{1,2}$ or epitopes shared between the two proteins (Mohan et al., 2012) allowing sGP to adsorb anti-$GP_{1,2}$ Abs. Another explanation could be sGP specific B cells are preferentially activated thereby expanding the repertoire and outcompeting $GP_{1,2}$ specific B cells, resulting in a humoral response biased towards sGP or shared epitopes with $GP_{1,2}$ (Figure 1.13 D). These two theories could explain why during acute infection there was no GP-specific Abs detected in patient sera, whereas Abs to other viral proteins, such as the NP (Feldmann, Klenk and Sanchez, 1999), were detected (Takada and Kawaoka, 2001). This could have implications if a person previously infected with the virus

was administered a vaccine encoding $GP_{1,2}$, potentially a non-protective immune response; Abs against sGP could be boosted (de La Vega, Wong, Kobinger, *et al.*, 2015).

1.9.2.1.3 sGP Lymphocyte apoptosis

Although the mechanism is unknown it has been suggested that sGP could be responsible for the bystander apoptosis of uninfected B and T cells not targeted for EBOV infection, due to its high abundance in the bloodstream (Figure 1.13 G). It has been reported that lymphocyte apoptosis in human EBOV infection can be mediated through death receptor ligand (Fas/ FasL) interactions (Wauquier et al., 2010). A continuation of the study assessed co-stimulatory effects of recombinant sGP on death receptor-induced ligand; TRAIL, FasL and TNF-α apoptosis in Jurkat cells. It was observed that there was no cell death of Jurkat cells (a T-lymphocyte cell line) in the presence of recombinant sGP (Wolf et al., 2011). Clarification is needed as to whether apoptosis of bystander cells is induced by sGP produced from EBOV infected cells (de La Vega, Wong, Kobinger, *et al.*, 2015).

1.9.2.1.4 sGP Neutrophil inactivation

There have been conflicting published results as to whether sGP has the ability to inactivate neutrophils. A study suggested sGP had the ability to bind and inactivate neutrophils through CD16b, a neutrophil-specific Fcγ receptor III; a key Fc receptor for recruitment of polymorphonuclear leukocytes following the binding of immune complexes (Figure 1.13 F) (Yang et al., 1998). This finding was later shown to be mediated via the binding between CD16b and the Fc portion of the anti-sGP Ab, which was confirmed when a Fab fragment failed to detect sGP binding to neutrophils (Sui and Marasco, 2002). This result argues against a direct interaction between sGP and neutrophil inactivation (de La Vega, Wong, Kobinger, *et al.*, 2015).

1.9.2.1.5 sGP Vascular dysregulation

It has been speculated that sGP could be responsible for changes to endothelial cells and vascular dysregulation observed during EBOV infection (Figure 1.13 E). A study found that in the presence of sGP there were no changes in cellular adhesion molecules (CAMSs), such as ICAM-1, VCAM-1 or E-selectin as well as VEcadherin arrangement, actin organisation or changes in transepithelial electrical resistance which indicates tight junctions between cells was not disrupted with the addition of sGP, thereby indicating no activation of endothelial cells or no influence on the endothelial cell barrier (Wahl-Jensen et al., 2005). A further

experiment showed a combination of TNF-α and sGP decreased barrier function, however, the addition of sGP partially restored activity, suggesting an anti-inflammatory role for sGP (Wahl-Jensen et al., 2005). Further research identified that the mutations Cys53Gly and Cys306Gly and double mutants impaired the sGP rescue of endothelial barrier function, Cysteine bonds are needed for sGP dimer formation indicating the sGP structure is important for this function (Falzarano et al., 2006). It is possible sGP may have a role to play in promoting vascular dysregulation, however, further research is required to support this statement.

1.9.2.1.6 Δ-peptide - Blocking receptor

The cleavage product formed from the C-terminus of pre-sGP (Δ-peptide) has been shown to be highly modified at the post-translational level (Volchkova et al., 1999). A study showed the peptide was able to inhibit EBOV and MARV virus entry into target cells by binding to unknown receptors (Miller et al., 2012) on filovirus permissive cells (Figure 1.13 I) (Radoshitzky et al., 2011).

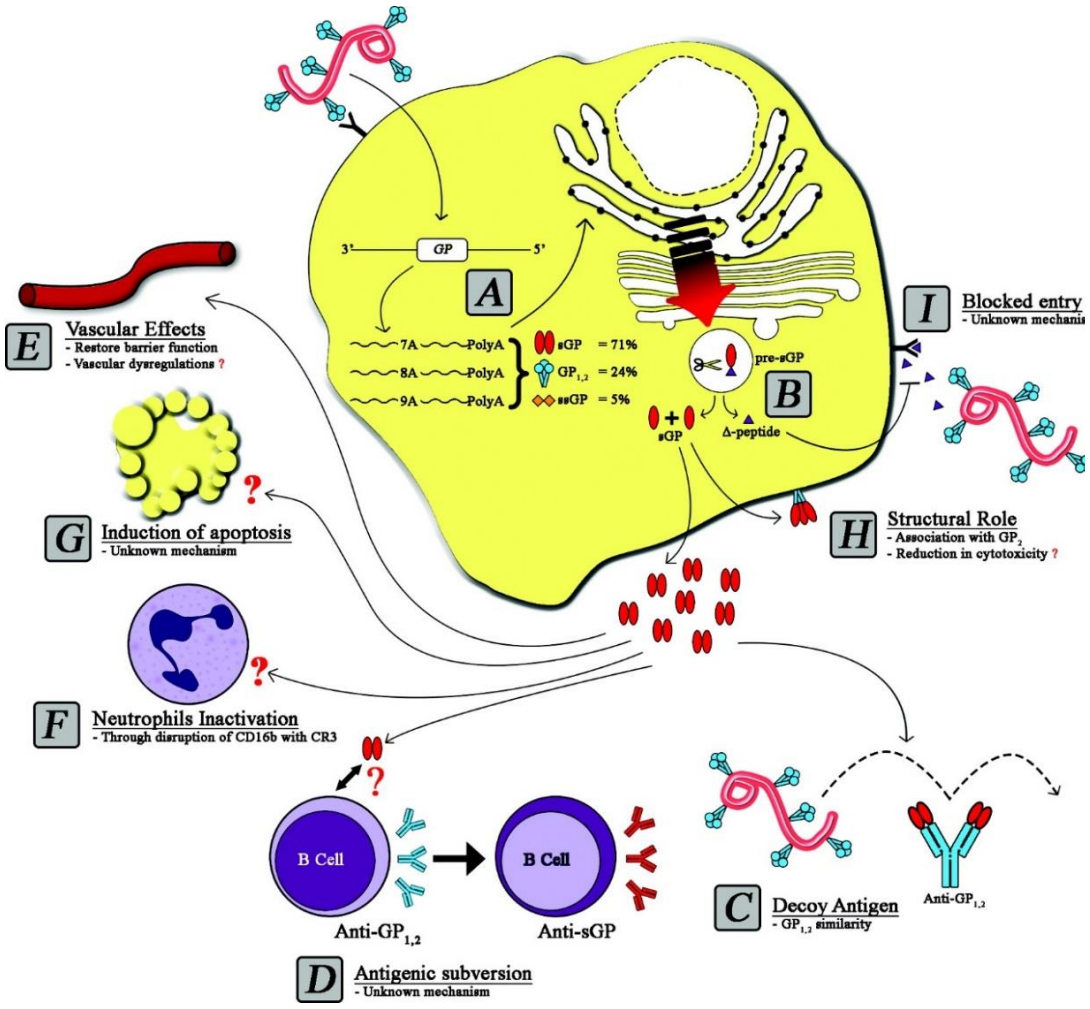


Figure 1.13 The multiple roles of sGP in EBOV pathogenesis

A) Post synthesis, pre-sGP, a trans golgi-specific precursor is cleaved by cellular proteases such as furin, yielding sGP and Δ-peptide. B) Monomers of sGP assemble as a homodimer in the cytoplasm and are secreted into the extra cellular medium. C) sGP may have a role as a decoy antigen, D) in antigenic subversion and E) restoring barrier function of endothelial cells. F) sGP may possibly have a role in inactivating neutrophils G) inducing apoptosis in bystander cells and H) have a structural role. I) Δ-peptide secreted from infected cells has been shown to be able to block the entry of filoviruses (Taken from de La Vega et al., 2015).

1.9.2.2 GP

The transmembrane form of the GP molecule is indispensable in EBOV pathogenesis and the viral life cycle. It is the only exposed protein on the virus surface and it is solely responsible for all stages of EBOV entry; binding, fusion and internalisation at the cell surface (Lee and Saphire, 2009; White and Schornberg, 2012) resulting in virus dissemination. $GP_{1,2}$ expression is cytotoxic to endothelial cells *in vitro,* causing cell rounding and detachment (Sullivan et al.,

2003; Xu et al., 1998) which may contribute to endothelial cell damage during EBOV infection. GP is the only Ebola gene product shown to have this effect and within the cell culture assay, the Zaire strain has been observed to be more potent (Simmons et al., 2002). This has been attributed to the mucin-like serine (S)- and-threonine (T) rich domain of GP which corresponds with down-regulation of specific molecules on the cell surface (Yang et al., 2000). These findings were mirrored in *in vivo* experiments, where replication-defective adenoviral vectors encoding GP or sGP were used to infect human blood vessel explant models and where, horseradish peroxidase (HRP) expression and EM revealed severe endothelial cell lining damage in vessels infected with GP but not sGP vectors. Likewise, EBOV strain specificity was shown when no toxicity was observed with Reston strain GP in human vascular explants, compared to significant tissue damage observed in the vascular explants of NHPs (Yang et al., 2000). GP-induced cytotoxicity is known to disrupt critical mediators of immune signalling such as the cell surface receptors, MHC class I proteins and several members of the β-integrin family. The mechanism is shared by several pathogens such as HIV-1 and used to evade host immune responses (Ploegh, 1998). Initially down-regulation or degradation of the cell surface proteins by EBOV GP was thought to occur, however, the EBOV GP mucin-like domain inhibits surface protein recognition by shielding surface epitopes (Francica et al., 2010; Lee et al., 2008; Reynard et al., 2009), representing a novel mechanism of disrupting immune function (Cook and Lee, 2013). Characteristics of EBOV infection; immune suppression, inflammatory dysregulation and loss of vascular integrity may largely be accounted for by GP induced biological effects (Sullivan et al., 2003).

Shed GP preserves its antigenic properties (Dolnik et al., 2015) and its described role is similar to that of sGP, it has been proposed that shed GP and sGP likely compete with the spike GP for Ab binding (William E Diehl et al., 2016; Ito et al., 2001). A guinea pig and monkey model for EBOV infection showed shed GP was present in the blood and served as a decoy for anti-GP Abs, including neutralising Abs (NAbs) inhibiting neutralising activity with EBOV Abs (Dolnik et al., 2004; Rubins et al., 2007). It has also been shown that shed GP triggers increased vascular permeability and an immune activation for systemic release of pro-inflammatory and anti-inflammatory cytokines contributing to its importance in EBOV pathogenicity (Escudero-Pérez et al., 2014). GP shedding plays a part in balancing optimal cell surface GP concentration, illustrating a control mechanism for GP-induced viral cytotoxicity, hence it may play an important role in EBOV replication (Dolnik et al., 2015).

1.9.2.3 ssGP

The current literature regarding the potential function of ssGP is limited. The structural and biochemical similarities between ssGP and sGP would propose a similar function between the two proteins. However a study conducted to determine if ssGP had an anti-inflammatory function as previously suggested and described for sGP, found no endothelial barrier rescue effect with ssGP and moreover no ssGP interaction with neutrophils was noted (Mehedi et al., 2011). The role of ssGP in EBOV pathogenesis remains unclear (Lee and Saphire, 2009).

1.10 Neutralising Abs

Neutralisation capacity is a measure of an Abs capacity to inhibit pathogen entry into a cell. Abs are considered neutralising when they assist in inhibiting the acquisition of a pathogen or limit its pathogenesis (Forthal, 2014) as well as targeting functionally active sites on pathogens. They are likely to be considered broadly neutralising Abs (bNAbs) if highly conserved regions of the pathogen proteins are recognised (Morris and Moody, 2016). One of the most important incentives for measuring NAb function is to provide a basis for vaccine development and for the development of therapeutics as well as preventing, reducing and clearing infection which serve as a correlate for most human vaccines (Forthal, 2014; Morris and Moody, 2016; Plotkin, 2013). A consideration of biological and medical importance is that neutralisation measured *in vitro* can be referred to how neutralisation may occur in an *in vivo* system (Klasse, 2014).

1.10.1 Mechanisms of neutralisation

Multiple mechanisms account for single Ab or Abs with different specificities which can neutralise an organism *in vitro,* generally occurring as a result of interference with an organism's attachment to host tissues (Forthal, 2014). Ab-Dependent Cellular Cytotoxicity (ADCC) and Ab-Dependent Cell mediated phagocytosis (ADCP) occur when an Ab forms a bridge between an Fc receptor (FcR)-bearing effector cell and an infected target cell, resulting in either lysis or apoptosis of the target cell or in the case of ADCC, internalisation and degradation of the Ab-coated pathogen by phagocytes (Forthal, 2014). Another mechanism, is the Ab activation of the complement cascade; composed of 30 proteins which can also result in lysis of infected cells or internalisation by phagocytic cells (Forthal, 2014; Spear et al., 2001). These Ab mediated mechanisms for neutralising pathogens are further

influenced by other factors. This includes the occupancy theories which suggests Immunoglobulin G (IgG) molecules because of their bulk impede the function of viral attachment; steric hindrance or alternatively Ab occupancy directly effects virus infectivity i.e. the fewer unoccupied functional entry-mediating molecules, the lower the infectivity (Klasse, 2014). Other mechanisms include the Abs ability to compete with receptor interactions of the virus, stoichiometry of Ab-mediated neutralisation, the breadth of epitope recognition (narrow type specificity versus broad cross-reactivity) and the affinity with which Abs bind to their respective virus epitopes (Vanblargan et al., 2016). Ab neutralisation potency is a compound phenotype that comprises of many complex mechanisms.

1.10.2 Serum Abs

Detectable serum Ab responses to a specific pathogen is usually a good indicator of circulating antigen-specific memory B cells and/ or plasmablasts from which mAbs can be isolated (Klein et al., 2013; Morris and Moody, 2016). For instance a large number of highly potent bNAbs to HIV-1 (Burton and Mascola, 2015) have been isolated by screening large volumes of donor sera for their capacity to neutralise viral isolates of multiple subtypes (Doria-Rose et al., 2010; Morris and Moody, 2016; Sather et al., 2009; Simek et al., 2009). Likewise the same approach was conducted for respiratory syncytial virus (RSV) and where healthy donors with assumed past infection were screened for serum activity against the virus (Corti et al., 2013; Morris and Moody, 2016). Broadly neutralising mAbs against influenza have largely been isolated from pandemic survivors (Wrammert et al., 2011; Yu et al., 2008), experimental and licensed vaccine recipients (Corti et al., 2010; Moody et al., 2011; Wrammert et al., 2008) and experimentally infected volunteers. Since antigen-specific Abs generally persist in the circulation of individuals for a period of time post disease onset; up to 40 years documented for EBOV survivors of the 1976 outbreak (Rimoin et al., 2018), serum or plasma can be screened long after infection (Moody et al., 2011; Morris and Moody, 2016).

1.10.3 Assays for Ab characterisation

Characterisation of Ab specificities responsible for serum neutralisation of pathogens, greatly aids efforts to isolate mAbs of interest. Due to NAbs being a small proportion of the polyclonal Ab response to pathogens, characterisation of the NAbs can be difficult and in order to overcome this, techniques such as peptide arrays have been used to screen Ab

specificities by analysing overlapping peptides. However, a shortfall to this approach are that NAbs recognise conformational epitopes and glycans that cannot be assessed by this strategy (Morris and Moody, 2016; Stephenson et al., 2015). The most widely utilised assays that consider the conformational epitopes and glycosylation of viruses, specifically EBOV when assessing NAbs in sera/ plasma generally fall into three categories: neutralisation of replication competent EBOV (RCE), neutralisation of EBOV PV and enzyme immunoassays (EIAs) (Mather et al., 2013; Wilkinson et al., 2017). The plaque reduction neutralisation test (PRNT) is the 'gold standard' assay for use with RCE. The donor sample is serially diluted, incubated with a standardised amount of virus and subsequently used to infect a confluent monolayer of permissive cells. After sufficient incubation for virus entry into cells, agarose or carboxymethylcellulose replaces the sample suspension, preventing virus released from infected cells to spread. Plaques of virus-induced cytopathic effect (CPE) are used to quantify virus infection. NAb is quantified by the patient sample reducing plaque formation by 50% ($PRNT_{50}$) or 90% ($PRNT_{90}$) compared to a plaque assay conducted with virus free from Abs (Mather et al., 2013). PRNT is used for NAb assessment, due to its high sensitivity and specificity. However, variations in protocols between labs has complicated international standardisation, the six or 12 well format and up to 6 day incubation for plaque visualisation limits throughput. The major drawback to the RCE PRNT is its restriction to biosafety level 4 laboratories (BSL4), of which there are only a few world-wide thereby limiting rapid serological screening (Mather et al., 2013). For this reason the PV system can be used in place of RCE for some research aspects.

Many variations of PV are available however they are based primarily on gene manipulation that encode for structural viral proteins in order to attenuate pathogenicity but maintain a serologically equivalent virus (Mather et al., 2013). A PV is a chimeric virion comprised of the structural and enzymatic core of one virus e.g. retroviruses such as HIV-1, expressing the GP molecule of another virus (e.g. EBOV GP) on its surface. Successful transduction of target cells by the PV is dependent on the ability of the GP to engage the receptors on the cell surface, leading to genome integration and expression of marker proteins, providing a quantitative read out of infection (Mather et al., 2013). Donor samples can be incubated with PV prior to infection of the target cell neutralisation by the Ab in the donor sample which can be quantified as a decrease in reporter gene expression compared with the PV transduction titre without Ab. The assay is advantageous due to there being no exposure to pathogenic

EBOV, can be performed with high throughput and it is widely available between laboratories (Mather et al., 2013).

Enzyme Linked Immunosorbent Assays (ELISAs) can be used with both RCE and PV, allowing quantitative measurements of Abs present in donor serum/ plasma sample that have bound to a viral protein. The 96 or 384-well ELISA format allows high throughput rapid screening. One drawback is that the Abs detected in the donor sample may not necessarily be Abs that are able to neutralise virus (Mather et al., 2013).

1.11 Commonly used packaging systems for enveloped PV

A number of packaging systems for enveloped PV have been investigated in relation to emerging viruses such as EBOV, the most commonly used systems are summarised below.

1.11.1 Retroviral Vectors

Retroviruses have an advantage over other viral systems, they are able to integrate foreign proteins, host-derived proteins and Env proteins of other viruses onto their envelope membrane (King and Tarr, 2017). These vectors are predominantly derived from HIV-1 and with the exception of Env proteins they retain all the genetic material required for viral transcription, packaging and integration. Retroviruses can be broadly classified according to their genome organisation into 'simple' and 'complex' viruses. Simple retroviruses comprise of *gag, pol* and *env* genes which encode the core proteins, viral enzymes and Env GP respectively, whilst complex retroviruses contain extra non-structural genes including the transcriptional activator *tat* (Weiss, 1996).

Simple retroviral packaging systems include the HIV-1, 2-plasmid system consisting of one Env expressing plasmid and the other the HIV-1 backbone i.e., pSG3$^{\Delta env}$ and pNL4-3 (the Env gene sequence in pSG3$^{\Delta env}$ is destroyed) (Figure 1.14) (Bosch and Pawlita, 1990).

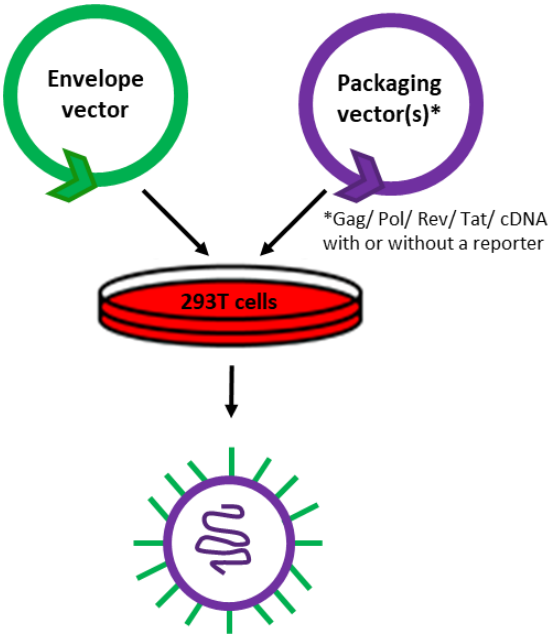


Figure 1.14 Retroviral PV packaging system

Simple HIV-1, 2-plasmid system. The packaging plasmid encodes HIV-1- gag, pol, rev, tat and cDNA and may encode for a reporter. The envelope expression plasmid encodes the glycoprotein (GP) of interest. Both plasmids are transfected into permissive cells such as 293T cells to produce pseudo-typed virus (PV) (Adapted from Addgene Lentiviral guide., 2018).

Another retroviral system commonly used for PV production is the murine leukaemia virus (MLV); its genome has been separated in 2: gag-pol and a reporter gene. Both of these genes were cloned into plasmids to generate efficient MLV packaging systems (Li et al., 2018). MLV retroviral packaging systems generally require nuclear membrane degradation during mitosis in order to integrate their genome into the host cell hence the virus can only infect actively dividing cells (Roe et al., 1993).

1.11.2 Lentiviral vectors

Lentiviral vectors fall under the category of complex retroviral systems and are widely used for packaging systems due to their high efficiency (Li et al., 2018). Lentiviral packaging systems are able to infect and integrate fully differentiated cells (King and Tarr, 2017). The HIV-1 packaging system involves specific genes cloned into DNA vectors, usually 3-4 plasmids are used as vectors.

The 3-plasmid system consists of plasmids for packaging, Env expression and transfer of the reporter gene. The HIV-1 proteins Gag and Pol are expressed in the packaging plasmid whilst the cis-regulatory elements needed for reverse transcription, integration, and packaging of HIV-1 are encoded in the transfer plasmid. Expression of the envelope gene is under the control of the cytomegalovirus (CMV) promoter within the expression plasmid (Figure 1.15). The 4-plasmid system is based on the 3-plasmid system with the HIV-1 REV protein encoded in a separate plasmid.

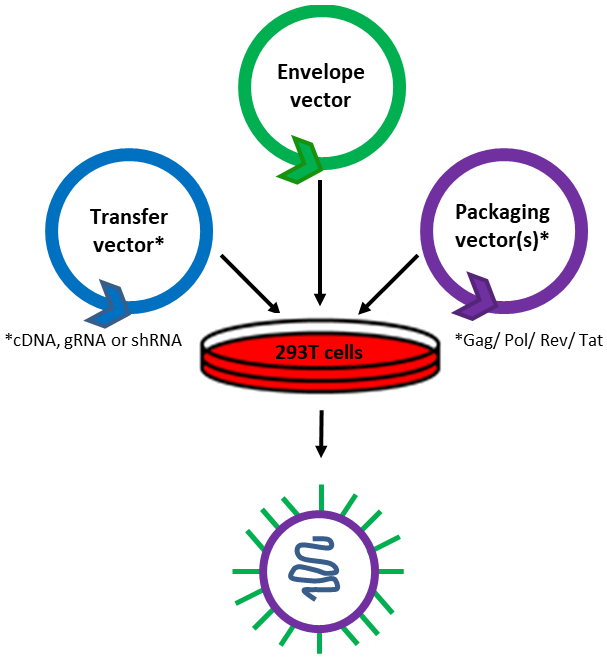


Figure 1.15 Lentiviral PV packaging system

HIV-1 3-plasmid packaging system. The packaging vector encodes the HIV-1 viral proteins gag, pol, rev and tat and may encode for a reporter. The transfer vector encodes cDNA whilst the envelope plasmid encodes the glycoprotein (GP) of the desired pseudo-typed virus (PV) (Adapted from Addgene Lentiviral guide., 2018).

1.11.3 The Vesicular Stomatitis Virus

The VSV system is advantageous in that it has no stringent selectivity for the Env proteins and several reporter genes have been inserted into this plasmid to facilitate its easy detection (Moeschler et al., 2016). However, it has been noted that PV produced with the VSV packaging system may contain residual VSV mixed with PV which can have implications if used in experiments such as neutralisation assays or by producing false positives. If an

interference by VSV is observed alternatives such as a VSV NAb may need to be utilised prior to use (Li et al., 2018). The VSV PV packaging system is depicted below (Figure 1.16).

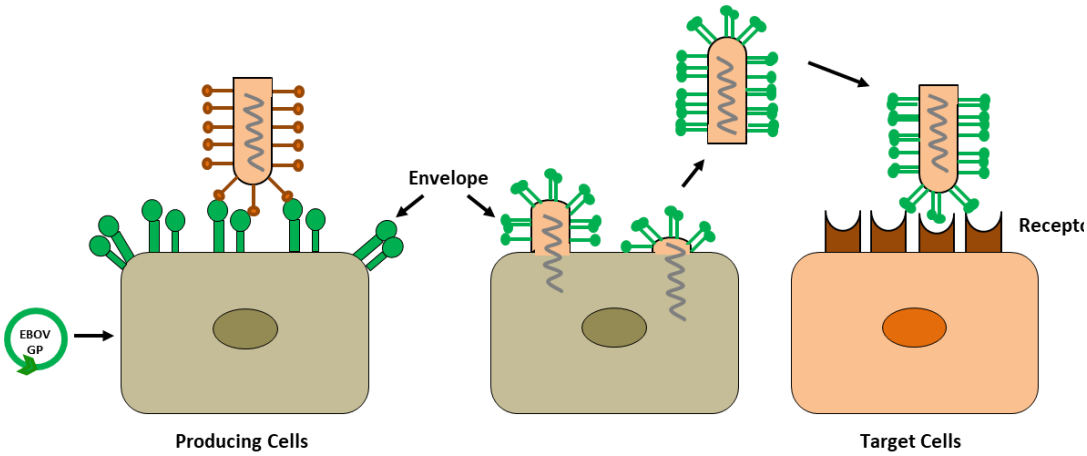


Figure 1.16 VSV pseudo-typed virus packaging system

An expression vector encoding the desired glycoprotein (GP) e.g. EBOV GP is stably transfected into a producer cell line, which subsequently expresses the EBOV GP. VSV is infected into the cell line and succeeding virions produced take up the EBOV GP on the virion surface. The VSV complemented with the EBOV GP Env is then used for infection into the target cell line (Adapted from Tani et al., 2012).

The packaging systems have permitted the production of many types of PV for different research purposes summarised below (Table 1.3).

Table 1.3 HIV-1, MLV and VSV Pseudo-typed virus packaging system

Summary of emerging viruses that have affected humans, the corresponding pseudo-typed virus (PV) system used to produce the enveloped virus and their subsequent use in research (Adapted from Li et al., 2018).

Packaging System	Virus Protein	Virus	Research area and application	References
HIV-1 VSV MLV	EBOV GP	Ebola virus	Virus entry/ inhibition Cellular tropism Glycoprotein function Neutralisation Ab assay Cell receptor recognition	(Kobinger et al., 2001; Quinn et al., 2009; Takada et al., 1997; Wool-Lewis and Bates, 1998)
HIV-1 MLV	HIV-1	Human Immunodeficiency virus	Cellular tropism Neutralisation Ab assay Cell receptor recognition Drug screening Virus inhibition	(Asin-Milan et al., 2014; Chan et al., 2006; Siegert et al., 2005; Wang et al., 2013; Zhao et al., 2016)
HIV-1 VSV	CHIKV	Chikungunya	Neutralisation Ab assay Env protein function Amino acid mutation of Env proteins on Cellular tropism Virus entry	(Hu et al., 2014; Kishishita et al., 2013; Salvador et al., 2009; Tong et al., 2015)

HIV-1	MARV GP	Marburg virus	Cellular tropism Virus entry	(Barrientos et al., 2004; S.Y. et al., 2000)
HIV-1	LASV GPC	Lassa virus glycoprotein precursor	Neutralisation Ab assay Conserved amino acid sequence of glycoprotein on fusion	(Klewitz et al., 2007; Robinson et al., 2016)
HIV-1 MLV	MERS-CoV	Middle East respiratory syndrome-coronavirus	Receptor validation, neutralisation, Ab evaluation Viral receptor amino acid domain Viral inhibition Cellular tropism	(Du et al., 2014; Grehan et al., 2015; Millet and Whittaker, 2016; Q. Wang et al., 2014; Yang et al., 2014; Zhao et al., 2013)
HIV-1 MLV	SARS-CoV spike	Severe acute respiratory syndrome-coronavirus	Cellular tropism Receptor recognition Cellular/ virus entry Neutralisation Ab epitope Gene transduction	(Han et al., 2004; He et al., 2005; Kobinger et al., 2007; Moore et al., 2004; Nie et al., 2004; Simmons et al., 2004)
HIV-1 VSV	NiV G/F	Nipah virus	Gene transduction Receptor recognition Neutralisation Ab assay	(Kaku et al., 2012, 2009; Negrete et al., 2005; Palomares et al., 2013; Tamin et al., 2009; Witting et al., 2013)

HIV-1 MLV	RRV E2E1A	Ross River virus	Cellular tropism Virus entry	(Kahl et al., 2005, 2004; Sharkey et al., 2001)
HIV-1	hV SH/G/F	Human respiratory syncytial virus,	Virus entry Neutralisation Ab assay Screening antivirus inhibitor Antivirus mechanisms	(Haid et al., 2016)
HIV-1	RV GP	Rabies Glycoprotein	Neutralisation activity of Ab Gene transduction Vaccine evaluation *in vivo/ vitro*	(Kato et al., 2007; Nie et al., 2017; Wright et al., 2008)
VSV	HCV GP E1/ E2	Hepatitis C virus	Env proteins in viral attachment and entry	(Matsuura et al., 2001)
VSV MLV	Hantavirus G1/G2		Neutralisation Ab assay Virus entry Cellular tropism Vaccine	(Higa et al., 2012; Lee et al., 2006; Millet and Whittaker, 2016; Ogino et al., 2003; Ray et al., 2010)
VSV	SFTSV G1/G2	Severe fever with thrombocytopenia syndrome virus	Infection diagnosis Drug screening Virus entry	(Tani, 2014)
VSV MLV	Arenavirus GPC		Receptor recognition Validate receptors	(Tani, 2014) (Kunz et al., 2005) (Tani et al., 2014)

VSV	JEV PrM/E	Japanese encephalitis virus;	Factors affecting viral infection and reproduction	(Tani et al., 2010)
VSV	MLV	Measles virus H/F	Neutralisation Ab assay	(Logan et al., 2016)
VSV	Lyssavirus GP		Neutralisation Ab assay	(Moeschler et al., 2016)
VSV	Avian influenza viruses HA/NA	HA hemagglutinin NA neuraminidase	Neutralisation Ab assay Antiviral screening	(Zimmer et al., 2014)
VSV	CCHFV GP	Crimean- Congo haemorrhagic fever virus	Virus entry	(Suda et al., 2016)
MLV	La Crosse virus G1/G2	G1/G2 Glycoprotein	Cellular tropism Neutralisation Ab assay	(Millet and Whittaker, 2016)
MLV	Visna virus Env		Cellular tropism	(Bruett and Clements, 2001)
MLV	VSV GP	Vesicular stomatitis virus	Transgenic study RNAi gene expression interference	(Kines et al., 2006; Yang et al., 2010)
MLV	Influenza virus HA	HA hemagglutinin	Screening specific Ab	(Wallerstrom et al., 2014; Wang et al., 2008)

1.12 Therapeutics against EBOV

Currently there are no licensed therapies against EBOV, neither for direct-acting anti-virals against EBOV infection or for the treatment of those suffering from EVD (Dhama et al., 2018). Due to the unprecedented scale of the 2013-2016 outbreak, the WHO prioritised the evaluation of experimental drugs and therapies on the basis of compassionate grounds, offering the potential to have a direct impact on the on-going outbreak whilst simultaneously determining clinical efficacy for various therapeutics (Espeland et al., 2018).

1.12.1 Passive Immunity

1.12.1.1 Convalescent plasma

Passive transfer of plasma from convalescent individuals has long been utilised in the treatment of certain disease outbreaks in humans (Garraud et al., 2016). Plasma comprises of a mixture of water, inorganic salts, organic compounds and more than 1000 proteins (Benjamin and McLaughlin, 2012). Immunoglobulins comprise a large part of the biomass of plasma (Benjamin and McLaughlin, 2012), however, other plasma components such as complement proteins and MBLs have also been found to be part of the plasma or serum, virus neutralisation response (Ji et al., 2005; Spear et al., 2001). The complement system, a key component of the innate immune response protects mucosal surfaces and is present in high concentrations in human plasma (Jayasekera et al., 2007). Viruses exposed to a source of complement can initiate complement activation and either directly mediate virus neutralisation by opsonisation or augment Ab-dependent neutralisation (Spear et al., 2001). MBL is a C-type lectin and one of its important innate immune function is direct neutralisation of some viruses by binding to carbohydrates expressed on some viral GPs including the filovirus envelope GP (Ji et al., 2005). However, in order to assess the specific reduction in viral infectivity by Ab binding, plasma is usually heat treated to inactivate interfering neutralising components during assays, utilising virus-neutralising plasma Abs (Jayasekera et al., 2007).

Plasma has been tested for its effectiveness in the treatment of EVD using NHP models and more recently human trials have also been conducted. An NHP model found macaques treated with convalescent whole blood, post EBOV challenge, succumbed to infection (Jahrling et al., 2007). Success was demonstrated when IgG was purified from the convalescent plasma (CP) of vaccinated NHPs that had survived an EBOV infection challenge; these Abs conferred protection against EBOV post exposure (Dye et al., 2012). However,

another study compared the ability of EBOV Makona specific sera to heterologous Sudan virus (SUDV) sera for the treatment of NHPs challenged with EBOV-Makona and demonstrated a decline of EBOV infection was not observed in NHPs and 100% protective benefit was not conferred (Mire et al., 2016). During the 2013 outbreak CP was donated from individuals who had cleared their EBOV infection and was administered to those suffering from EVD (Zeitlin et al., 2016). This was the first time CP had been used in a large epidemic setting for EBOV, as previous uses were limited to a handful of patients, such as during the 1995 Kikwit EBOV outbreak (Mupapa et al., 1999).

Three CP trials enrolling EVD patients were initiated during the outbreak (Colebunders and Cannon, 2015). The most comprehensive trial, the Ebola-Tx trial analysed 84 EBOV infected patients who received two consecutive transfusions of 200-250 ml ABO-compatible CP with unknown neutralising titres and each from a separate donor (van Griensven, Edwards, de Lamballerie, Semple, *et al.*, 2016). Although the trial results showed mortality was lower in the CP group compared to the controls the differences were not significant. A follow up study taking into account age and baseline cycle threshold (Ct) values showed higher IgG doses resulted in lower mortality, however, a higher level of NAbs also resulted in higher mortality, although neither association reached statistical significance (van Griensven et al., 2016a). It was concluded that the inability to determine NAbs levels in CP was a limiting factor (van Griensven et al., 2016b), Ab titres in human CP tend to be low and there is variation in Ab titres between different batches of plasma pools (Cardile et al., 2017). If titres of NAbs are a key factor in CP therapeutics, then an Ebola-GP PV assay developed for on-site use would have been highly beneficial in screening CP for activity (Cardile et al., 2017).

1.12.1.2 Vaccines

One means of overcoming insufficient NAbs titres is via the use of passive vaccines for the treatment of EVD. As the GP is the target of many NAbs, a study which mapped the functional mAb epitopes of the EBOV GP (Ponomarenko et al., 2014), is summarised below (Table 1.4). These epitopes were experimentally identified and were targets of Abs that were found to be protective against the Zaire EBOV strain. The study found that protective mAb epitopes in the Ebolavirus GP sequences within the Zaire EBOV lineage, since 1976 was highly conserved which bode well for the general usefulness of these Abs with regards to the 2013 EBOV outbreak (Ponomarenko et al., 2014).

Table 1.4 Description of mAbs targeting the EBOV GP

Protection indicates a positive live *in vivo* survival or challenge assay.
VN denotes virus neutralisation assay
Adapted from (Ponomarenko et al., 2014)

Epitope sequence	Antigen (epitope positions as in IEDB)	PMID for epitope record	mAb	EBOV species	Functional Assay*
GKLGLITNTIAGVAGLI	GP_1 (477-493)	22171276	**14G7**	Zaire ebolavirus	Protection against EBOV in mice [PMID: 10698744]
AGNNNTHHQDTGEESASSGKLGLITNTIAGVAGLITGGRRTR	GP_1 (459-500)	21925951	**4G7 (ZMAb)**	Zaire ebolavirus	Protection against ZEBOV in mice
VEQHHRRTD	GP_1 (404-412)	18005986	**13F6-1-2 (MB-003)**	Ebola virus - Mayinga, Zaire, 1976	Protection against EBOV in mice [PMID: 10698744]
VYKLDISEA	GP_1 (393-401)	10698744	**6D8 (MB-003)**	Mayinga, Zaire, 1976	VN, Protection
GP1: V42, L43; GP2: V505, N506, A507, Q508, P509, K510, C511, N512, P513, N514, H549, N550, Q551, D552, G553, L554, I555, C556	GP_1-GP_2	18615077	**KZ52**	Ebola virus - Mayinga, Zaire, 1976	VN
H549	GP_2	12502822	**133/3.16**	Zaire ebolavirus	VN, Protection
R134, F194, L199	GP_1	12502822	**226/8.1**	Zaire ebolavirus	VN, Protection

A promising Ab candidate ZMapp™ was developed as an optimised combination of three humanised mAbs from the two cocktails ZMAb and MB-003 (Qiu et al., 2014). ZMapp™ demonstrated 100% protection in rhesus macaques administered as late as five days post infection (dpi) after an intramuscular (IM) EBOV challenge with a lethal dose, with reversion of EVD also described (Zeitlin et al., 2016). The PREVAIL II study evaluated the efficacy of ZMapp™ with a total of 72 enrolled patients; half received optimal standards of care (oSOC)

whilst the rest received oSOC and ZMapp™. Overall statistical significance of administering ZMapp™ with oSOC was not reached, however, mortality was 40% lower in the ZMapp™ group compared to the control (Liu et al., 2017).

The 2013-2016 Ebola outbreak accelerated the clinical evaluation of two vaccines, rVSVΔG-ZEBOV-GP (V920) and Ad26-ZEBOV/MVA-BN Filo prime-boost. V920 is a recombinant, replication competent VSV based vaccine expressing the Zaire EBOV GP (Henao-Restrepo et al., 2015). Previous testing in cynomolgous macaques conferred 100% protection against a lethal challenge of Zaire EBOV 28 days post vaccination, likewise macaques challenged with Makona variant of EBOV 7 days post vaccination also survived. A ring vaccination approach was implemented to contacts and contacts of contact during the 2013-2016 epidemic, when a newly confirmed case of EVD transpired (Henao-Restrepo et al., 2015). The V920 vaccine was documented to stimulate a rapid onset of IgG and NAbs that were sustained up to one year post vaccination (Heppner et al., 2017).

The Ad26-ZEBOV/MVA-BN Filo prime-boost vaccine is comprised of an adenovirus type 26 vector vaccine encoding EBOV GP and a modified vaccinia virus Ankara vector vaccine encoding GP from Ebola, Sudan, Marburg and Tai Forest viruses NP (MVA-BN-Filo). NHP studies demonstrated 100% efficacy when either Ad26- or MVA- vectored vaccines were administered with subsequent boosting by the alternative vector vaccine encoding the same EBOV GP (heterologous prime/boost) (Krause et al., 2015; Milligan et al., 2016). The human trial was documented to be safe and well tolerated producing sustained immune responses up to one year post vaccination (Espeland et al., 2018; Milligan et al., 2016; Winslow et al., 2017). The study concluded that although no correlate of protection was established, EBOV GP-specific Abs appear to play an important role in immunity (Dye et al., 2012; Winslow et al., 2017).

1.12.2 Non-Ab based therapeutics

Various non-Ab based therapeutics have been tested in the treatment of EVD during the 2013 outbreak. Direct-acting antivirals, nucleoside and nucleotide analogues are compounds that can exert antiviral effects via inhibition of viral polymerases, enzymes such as kinases and/or incorporation into viral nucleic acids (Cardile et al., 2017; Jordheim et al., 2013) an example being Favipiravir which inhibits the RdRp (Cardile et al., 2017; Furuta et al., 2013; Madelain et al., 2017).

Nucleic acid based therapeutics including antisense phosphorodiamidate morpholino oligomers (PMOs) and small interfering RNAs (siRNAs) have been assessed for the treatment of EVD. TKM-130803 is a nanoparticle formulation and is comprised of three siRNAs and targets RNA transcription and replication components; L polymerase and EBOV antigens VP24 and VP35 (Volchkov et al., 1999).

1.13 Aims

Based on the advantages and disadvantages of the aforementioned assays, specifically the ease and high throughput permitted with screening EBOV survivor CP with variants of EBOV, we elected to proceed with a PV neutralisation assay for our study. The development of a PV assay would allow us to:

- Establish a well-characterised assay permitting the measurement of EBOV virus strain infectivity without requiring a high containment laboratory.
- Screen and analyse the neutralising properties of CP from a cohort of Ebola survivors.
- Determine the evolution of EBOV NAbs over time in the cohort of survivors.
- Evaluate the interaction of sGP secreted EBOV protein to interact with the CP of Ebola survivors.

Overall, the aims of this study are towards understanding the role EBOV GP specific NAbs have on inhibiting EBOV entry and determining the implications for future vaccine design aimed at preventing or controlling EBOV infection.

2 Materials and Methods

2.1 Plasmid Preparation

2.1.1 Bacteria transformation

Plasmids were transformed into chemically competent *E. coli* using One Shot® TOP10 cells (Thermo Fisher). One vial of cells were thawed on ice then 2 µl of plasmid stock was added, tapped gently to mix and incubated on ice for 30 min. The cells were subjected to a heat shock at 42°C for 30 s in a water bath; creating pores in the bacterial membrane allowing the plasmid DNA to enter the cells. This was promptly followed by 30 s on ice, the addition of 250 µl of S.O.C and incubation at 37°C for 1 h at 225 revolutions per minute (rpm). S.O.C, is a rich medium that aides in the recovery of the transformed bacteria and increases transformation efficiency of competent cells. The transformed bacteria were diluted 1/50-1/100 before being plated on antibiotic selective agar plates which were left at 37°C overnight (O/N).

2.1.2 Bacteria inoculation

Following successful growth of the transformed bacteria on the agar plates, aseptic technique was followed and colonies were picked and inoculated into 5 ml of ampicillin/ BHI media. Bacteria were left to grow at 37°C, 225 rpm in a shaking incubator for 6-8 h. 1 ml of the turbid cultures; an indication of bacteria growth was used to generate glycerol stocks in a 1:1 ratio with glycerol (Sigma-Aldrich) and frozen at -20°C before storage at -80°C. The remaining culture was either centrifuged at 6000 xg for 10 min at 4°C for small scale plasmid preparation by the miniprep kit (Qiagen) or 1 ml of the culture was used to inoculate 500 ml ampicillin/BHI media for large scale bacteria growth for use in maxipreps (Qiagen).

2.1.3 DNA plasmid isolation

The Miniprep and Maxiprep kits (Qiagen) were used to isolate and purify DNA from the bacteria culture; following the manufacturer's instructions. Plasmid DNA stocks were eluted in sterile deionised water and were prepared with Maxi prep kits as they yielded a higher volume of DNA. The concentration and purity of the DNA isolated was checked using a nanodrop (Thermo Scientific). The wavelength of maximum absorption for both DNA and

RNA is 260 nm therefore an absorbance ratio of approximately 1.8 at 260/280 nm indicates a relatively pure DNA sample.

2.2 Cell culture methods

2.2.1 Cultivation of HEK-293T, TZM-bl, Huh7 and Vero cells

HEK-293T cells containing the SV-40 T antigen were derived from the Human Embryonic Kidney 293 cells and is widely used as a highly transfectable cell line. TZM-bl is an indicator cell line derived from a HeLa cell clone and contains a HIV-1 Tat induced firefly luciferase reporter gene, allowing for sensitive and quantitative measurements of infection. Huh7 is a hepatocyte carcinoma derived cell line and Vero are derived from the kidney of African green monkey. HEK-293T, TZM-bl, Huh7 and Vero cells are adherent cell lines and were cultivated in Dulbecco's modified eagle medium (DMEM; Invitrogen), supplemented with 10% heat-treated foetal bovine serum (FBS) (Sigma), 2mM/ml L-glutamine (Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogn). Cells were grown in a humidified atmosphere at 37^{o}C and 5% $CO_{2.}$ Once cells reached 70-80% confluence they were passaged routinely every 3-4 days. Cells were washed with 5 ml PBS, incubated for 5 min with 2 ml 0.25% Trypsin-EDTA solution at 37^{o}C and 5% $CO_{2.}$ Detached cells were re-suspended in 12 ml of fresh cell culture medium, 1 ml of the cell suspension was transferred to a new flask with 11 ml of fresh cell culture medium to continue the cell line and the remaining cells were either counted in preparation for cell seeding (see cell counting protocol) or discarded.

2.2.2 Cell counting

Trypsinised cells were re-suspended in 12 ml of cell culture medium. Re-suspended cells and trypan blue (Sigma-Aldrich) were mixed in a 1:1 ratio which was subsequently pipetted onto a Bright-line haemocytometer (Sigma-Aldrich) in order to fill the chamber with cell suspension. Viable cells were counted on the grid as per manufacturer's instructions and the following calculation was used to calculate the cell number per ml of medium:

(Viable cells ÷ 4) x2 (dilution factor) x 10^4 = cells/ ml in cell suspension

2.2.3 Cell seeding

Cell lines for PV transfection and infection were seeded 1 day prior to use. Post cell counting, cells to be used for the propagation of PV were seeded at a density of 1.2 x 10^6 cells in 7 ml of DMEM complete media in 10 cm tissue culture treated dishes (Corning). Cells used for infection were seeded in a white tissue culture treated 96 well plate (Corning) at a density of 15 x 10^3 cells/ well in 250 µl DMEM complete media.

2.3 Virological Assays

2.3.1 EBOV PV construct design

The five EBOV PV constructs comprising of the surface GP were designed by G. Pollakis, University of Liverpool. The strains of the PV designed were: the 2013-2016 epidemic strain (M-14), the 1995 Kikwit strain (K-95), 2014 DRC strain (L-14), and two mutant 2013 epidemic strains. The 2013-2016 epidemic strain used in this study was based on the first full EBOV sequences, which were nearly identical, from three patients of the epidemic (Baize et al., 2014). An analysis of sequenced EBOV strains between March-August 2014 revealed mutations that were only observed once (MSEM-14) and mutations that arose multiple times (MEM-14). These mutations were incorporated into the 2013 epidemic strain sequence respectively. The EBOV strains used in this study are shown in the alignment (Figure 2.1) the consensus sequence (K1660348) used was one of the first, full patient EBOV sequences published (Baize et al., 2014). The yellow highlighted letters indicate an amino acid difference in the GP regions of the respective EBOV strain compared to the consensus sequence at a specific location.

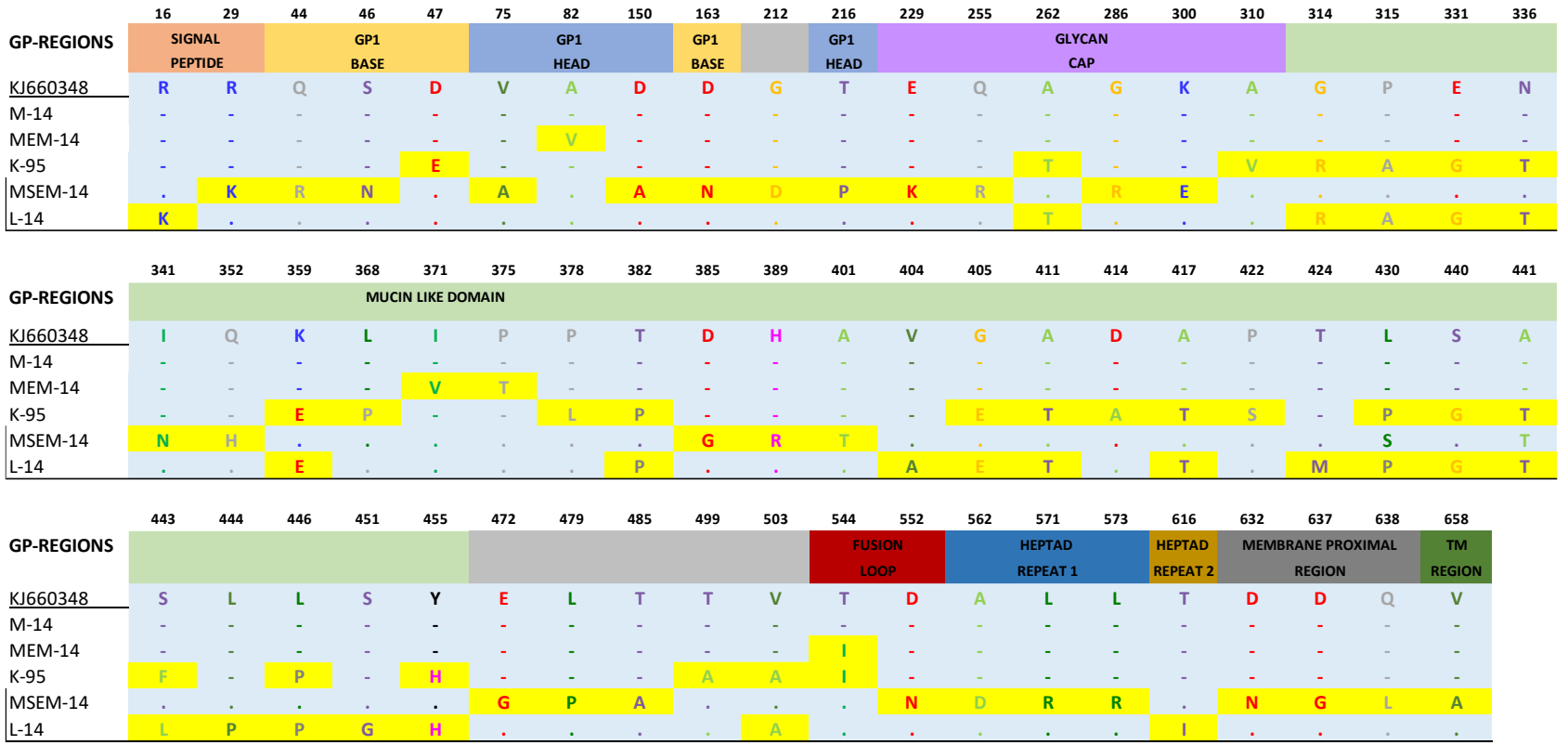


Figure 2.1 EBOV GP amino acid sequences of virus strains used for the PV constructs

Alignment of the amino acid sequences in the GP region for the five strains of EBOV PV used in this study; 2013 epidemic strain (M-14), two 2013 mutant strains comprising of either multiple (MEM-14) or singular mutations (MSEM-14) that arose early in the EBOV epidemic, 1995 Kikwit strain (K-95) and the 2014 DRC strain (L-14). EBOV strains were aligned to a reference strain; K3660348, one of the first, full EBOV sequences of the 2013 epidemic (Dots indicate sequence similarity and letters, a modification in the amino acid sequence for that strain at that specific amino acid position compared to the reference strain, highlighted in yellow).

2.3.2 Preparation of Polyethylenimine (PEI)

Polyethylenimine (PEI) (Polysciences cat no 23966) used in the transfection protocol was prepared in 150 mM NaCl to a final concentration of 1 mg/ ml. The PEI was dissolved by incubating the solution in an 80°C water bath, swirling intermittently to aid in dissolving the PEI. Once the PEI was dissolved the reagent was cooled to R.T before adjusting the pH to 7.0. The PEI was stored at 4°C, kept for a maximum of 3 months and passed through a 0.2 µm filter prior to use.

2.3.3 Pseudo-typed viral production by transfection

A pseudo-typed retroviral system was used to routinely generate single-cycle infectious viral particles through transfection. Two plasmids are typically co-transfected into a producer cell line, one expressing the proteins required for virus generation and the other the protein envelope of choice to be studied. The envelope GP of PV is crucial for entry into target cells therefore manipulation of the envelope proteins is one way in which to study virus entry. An expression plasmid encoding the sequence of EBOV-GP strain M-14, MEM-14, K-95, L-14, MSEM-14 as well as the EBOV secreted soluble protein (sGP), the HIV-1 envelope protein, LAI (utilising the CXCR4 co-receptor; NIH AIDS reagents repository) and VSVg (a non-tropic envelope; NIH AIDS reagents repository) were co-transfected with an envelope deficient HIV-1 backbone either pSG3$^{\Delta env}$ (NIH AIDS reagents repository) or pNL4-3.Luc.R-E (encoding a firefly luciferase reporter; NIH AIDS reagents repository). The pSG3$^{\Delta env}$ and pNL4-3.Luc.R-E plasmid contain the genes required for virus production whilst the plasmids encoding the envelope proteins are necessary for providing infectious particles and viral entry into host cell lines. Following transfection of the producer cell with a cationic polymer transfection reagent (PEI) in the presence of OptiMEM (Invitrogen) the cells are able to express both the viral and envelope proteins subsequently generating PV (Figure 2.2). Further generations of infectious virus cannot be produced as the incorporated viral genome of the produced virus lack the envelope gene and therefore cannot generate new infectious particles.

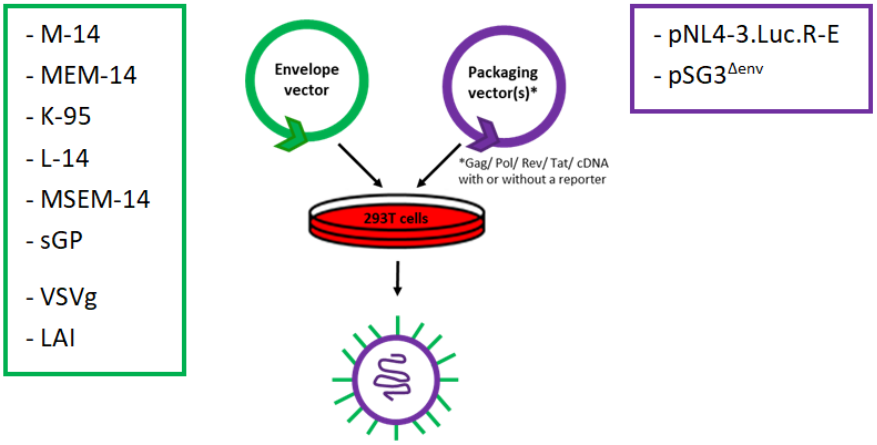


Figure 2.2 Retroviral PV packaging system

The packaging plasmid (purple) encodes HIV-1- gag, pol, rev, tat and cDNA. In this study pSG3$^{\Delta env}$ which does not encode a reporter as well as pNL4-3.Luc.R-E which encodes for a luciferase reporter was utilised. The envelope expression plasmid (green) encodes the glycoprotein (GP) of interest. In this study the EBOV strains; 2013 epidemic strain (M-14), two 2013 mutant strains comprising of either multiple (MEM-14) or singular mutations (MSEM-14) that arose early in the EBOV epidemic, 1995 Kikwit strain (K-95) and the 2014 DRC strain (L-14) were utilised as well as the EBOV sGP; Makona 2014 strain and control envelope proteins; VSVg and HIV-1 LAI. One of the two packaging plasmids was transfected alongside an envelope expression plasmid into permissive 293T cells to produce pseudo-typed virus (PV) (Adapted from Addgene Lentiviral guide 2018).

Specifically 0.285 µg of the envelope plasmid (unless otherwise stated) and 2 µg of pSG3$^{\Delta env}$ were mixed in 300 µl of OptiMEM in an eppendorf tube. OptiMEM and PEI were also mixed together; 276 µl and 24 µl respectively with 300 µl added to the DNA mix, vortexed briefly and left to incubate for 30 min at R.T. Meanwhile the media of the 293T cells in the 10 cm TC dish was replaced with 4 ml of OptiMEM and post incubation 600 µl of the DNA mix was added drop by drop to the cells. TC dishes were incubated at 37°C, 5% CO_2, 6 h later OptiMEM was replaced with 8 ml of complete DMEM. 72 h post-transfection supernatant containing PV particles were harvested, passed through a 0.45 µM filter and stored in aliquots at −80°C.

2.3.4 Pseudo-typed viral infection

Infections were carried out on 293T, Vero and TZM-bl cells with PV produced with the pNL4-3.Luc.R-E backbone and on the TZM-bl cell line with virus containing the pSG3$^{\Delta env}$ backbone. The TZM-bl luciferase activity (expressed from the LTR promoter) is under the control of Tat expressed from the HIV-1 backbone.

A volume of 100 µl of PV; pSG3$^{\Delta env}$ used as a negative control, pSG3$^{\Delta env}$ VSVg; a positive control and pSG3$^{\Delta env}$ EBOV-GP was used to infect 1.5x10^4 TZM-bl cells/ well in a 96 well plate (Corning). The plates were incubated at 37°C, 5% CO_2 and 6 h later 150 µl/ well DMEM complete was added to the cells. The plates were then incubated for 48 h prior to luciferase measurement.

2.3.4.1 Pseudo-typed viral infection with DEAE-Dextran

To measure the effect of Diethylaminoethyl (DEAE)-Dextran on the infectivity of the PV, infection was carried out as previously described (2.3.3), using virus stocks produced with pNL4-3.Luc.R-E and EBOV-GP; MSEM-14, L-14, K-95. 100 µl of virus stocks were used to infect 1.5x10^4 293T, TZM-bl and Huh7 cells in a 96 well plate. 6 h post- infection DMEM complete media containing 30 µg/ml Dextran was added to the cells. 48 h post- infection luciferase activity was measured.

2.3.4.2 Pseudo-typed viral infection with soluble glycoprotein (sGP)

The infectivity of PV incubated with sGP both produced in-house; Makona 14 strain and commercially available; 1995 Kikwit strain, was evaluated. Virus stocks were produced with only the pSG3$^{\Delta env}$ and EBOV-GP M-14 and K-95 strains. Infection was carried out as previously described onto TZM-bl cells in a 96 well plate, sGP concentrations incubated with virus stocks prior to infection are stated in individual experiments.

2.3.5 Measuring luciferase activity

48 h post infection supernatant was discarded from the wells, cells were washed with phosphate buffered saline (PBS) and lysed with 50 µl cell lysis buffer (ThermoFisher) or 30 µl cell lysis buffer (Promega). After 15 min incubation, luciferase activity was measured using either the Firefly Luciferase Glow Assay (ThermoFisher) or Luciferase Assay kit (Promega) and a FLUOStar Omega luminometer (BMG LabTech) according to the manufacturer's instructions. The Firefly Luciferase Glow Assay (ThermoFisher) was used for one assay where stated whilst all other times the Luciferase Assay Kit (Promega) was used.

2.3.6 Virus quantification (p24 capsid ELISA)

Virus quantification was carried out using the p24 antigen sandwich Enzyme Linked Immunosorbent Assay (ELISA) using Aalto Bio Reagents Ltd protocol. Samples were diluted in 0.1% Empigen/ TBS prior to input on the ELISA plate which was pre-coated with the p24

coating Ab (polyclonal sheep anti-HIV-1-p24 gag) allowing capture of the p24 antigen. The secondary conjugate (alkaline phosphatase conjugate of mouse monoclonal anti-HIV-1-p24) was added, which also binds to the p24 antigen. Plates were washed with ELISA light assay buffer (Thermo Fisher scientific) before ELISA light (CSPD with Sapphire Substrate, Thermo Fisher scientific) was added. Plates were incubated for 30 min at R.T prior to measuring luminescence with FLUOStar® Omega luminometer (BMG LabTech).

2.3.7 Plaque Reduction Neutralisation Test (PRNT)

This work was carried out by R. Gopal, PHE.

The strain used for assays with RCE was EBOV Makona C7 (GenBank accession number KJ660347.2), isolated from a female Guinean patient in March 2014 (virus provided to PHE Porton by Stephan Günther, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany). The virus was cultivated in Vero E6 cells and virions in the supernatant were pelleted by ultracentrifugation through a 20% glycerol cushion. A 10,000-fold dilution of a preparation with an infectious virus titre of 10E9 focus forming units per ml were used for coating both Indirect and blocking EIA microplates (Jacobs et al., 2016).

2.4 Ab Assays

2.4.1 Ebola survivor cohort

Ebola survivors were previously recruited for the study 'Convalescent plasma (CP) for early Ebola virus disease in Sierra Leone". The study (ISRCTN13990511 & PACTR201602001355272) was approved by the Scientific Review Committee and Sierra Leone Ethics, authorised by the Pharmacy Board of Sierra Leone (PBSL/CTAN/MOHSCST001) and sponsored by the University of Liverpool (R.S. Tedder et al., 2018). CP samples were subsequently used in our study.

2.4.2 Cohort follow up questionnaire

A follow up questionnaire was given to selected individuals of the Ebola survivor cohort February 2018. The questions included:

At the time when you were donating plasma for Ebola_CP (2015) were you given any vaccine?

Yes/ No

At the time when you were donating plasma for Ebola_CP (2015) were you part of an Ebola Vaccine study?
Yes/ No

2.4.3 Plasma preparations

Whole blood samples from donors were collected and separated into blood components within 24 h of venesection. Plasma samples received by Public Health England (PHE) Colindale were tested for EBOV RNA and confirmed negative. Following the UK Ebola survivor case where re-emergence of the virus was seen, all plasma samples were re-tested for EBOV RNA prior to aliquoting and usage.

Upon arrival at the University of Liverpool, the plasma samples were stored at -80°C. Samples selected for our study were thawed in a 37°C water bath for 10 min before plasma was aliquoted into cryovials for storage at -80°C. Prior to use, plasma was complement inactivated at 56°C for 30 min and centrifuged at 10,000 rpm for 5 min to pellet plasma debris. The supernatant was further aliquoted into volumes of 20 µl and stored at -80°C.

2.4.4 Double Antigen Bridging Assay (DABA)

The DABA analysis was conducted by Prof. R. Tedder, PHE. DABA is an enzyme linked immunoassay which was used to quantify EBOV GP targeting Abs present in Ebola survivor CP samples. An EBOV GP antigen, Mayinga Zaire EBOV strain (IBT Bioservices) was pre-coated onto the 'solid phase' (usually a polystyrene microwell strip) which acted as a ligand to EBOV Abs that may be present in the human serum/ plasma sample. A detector; a second antigen conjugated to HRP was added, if EBOV Abs were present in the sample then both antigens bound to the variable domains of the Ab leading to a specific, antigen-Ab-antigen-HRP immune complex which was bound to the solid phase (Figure 2.3).

The Ab titres were expressed as arbitrary units/ml (au/ml) as compared to a standard; five reactive donor samples that were pooled and attributed 1000 au/ml. The addition of TMB to the complex hydrolysed the colourless chromogens turning it blue, the amount of colour measured in the wells was proportional to the amount of Ab present in the sample (R.S. Tedder et al., 2018).

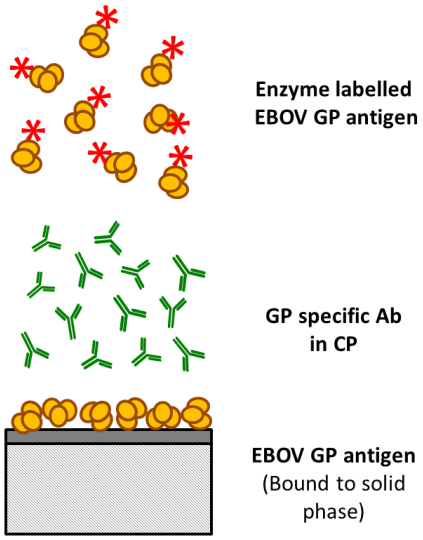


Figure 2.3 Double Antigen Bridging Assay (DABA)

Schematic diagram of the quantitative Enzyme Immune Assay (EIA), DABA. EBOV antigen Zaire ebolavirus GP is bound to the solid phase allowing immobilisation of anti-EBOV GP Abs present in convalescent plasma/ serum of Ebola survivors. A secondary EBOV antigen conjugated to HRP is added, binding to the free variable domain of the Ab. A successful antigen-Ab-antigen immune complex is indicated with a colour change after the addition of TMB. The degree of colour change is proportional to the amount of anti-EBOV Abs present in the sample.

2.4.5 Pseudo-typed virus Neutralisation Assays

A panel of Ebola survivors CP (n= 102) as well as plasma from healthy volunteers (n= 6) were serially diluted ½ with DMEM and 13 µl plasma dilution was incubated with 200 µl EBOV-GP PV for 1 h at R.T. 100 µl of virus/plasma dilution was used to infect TZM-bl cells as described previously. Luciferase readings of neutralised virus were expressed as the reciprocal of the highest dilution. The neutralisation potential of a CP sample was represented as the plasma dilution that reduced viral infectivity by 50% (IC_{50}).

2.4.6 Pseudo-typed virus Neutralisation Assays with sGP

The effect of sGP on CP neutralisation with EBOV virus stocks was assessed. Based on the pre-determined IC_{50} titre for a particular CP sample, the corresponding plasma dilution was carried out and a specific amount of sGP (stated in individual experiment) was added to the mix prior to addition of the virus. Infection into TZM-bl cells was carried out as previously described. sGP utilised in these experiments were either produced in-house or commercially produced; subtype Zaire strain Kikwit 1995 (GP- 634E 2BSCIENTIFIC LTD).

2.4.7 Enzyme Immune Assay (EIA)

The indirect and blocking EIA was conducted by Dr R. Gopal, PHE.

2.4.7.1 Indirect EIA

An Indirect EIA was carried out using 384 well microplates to determine total anti-EBOV IgG of CP by reactivity of serial plasma dilutions against EBOV. Bound human IgG was detected with a specific anti-human HRP conjugate (DAKO) (Jacobs et al., 2016).

2.4.7.2 Blocking EIA

A blocking EIA was conducted with EBOV CP Abs to EBOV GP, VP40, NP to determine the ability to block the binding of specific rabbit anti-peptide (GP, VP40, NP) and anti-VLP (Zaire EBOV) Abs (IBT Bioservices) to EBOV Makona virion coated microplates. In brief, EBOV patient CP and negative plasma serum dilution (1/100) were plated onto virion coated microplates for 4-6 h. Plasma was removed and plates were then incubated with EBOV specific rabbit Abs. Bound rabbit Abs were detected by specific anti-rabbit HRP conjugate (DAKO). EBOV protein specific Abs present in CP were determined by the reduction in the binding of the corresponding rabbit Ab in comparison to the negative control read out. Results were expressed as a percentage reduction of the negative control binding (Jacobs et al., 2016).

2.5 Protein Assays

2.5.1 Protein concentration

EBOV soluble glycoprotein (sGP); strain Makona 2014 was produced from transfection of 293T cells (described in 2.3.3) and was concentrated with the Amicon®Ultra-15 centrifugal filter device (Merck Millipore, UK) with a 30,000 Molecular Weight Cut off (MWCO). The protocol followed was as per manufacturer's instructions; in brief 15 ml of supernatant containing protein was transferred to the filter device and centrifuged using a swing-bucket rotor at 4,000 x g for approximately 15-60 min. The ultrafiltrate collected in the centrifuge tube was removed and stored for use as a control in the protein quantification assay. The 200 µl of concentrate in the filter device was reconstituted with 14.8 ml PBS to exchange out the culture medium and the filter was centrifuged again, these steps were repeated twice. The concentrated protein was aliquoted and stored at -20°C.

2.5.2 Bradford Assay

The Quick Start™ Bradford Reagent (Bio-Rad) was used for quantification of total protein concentration in transfected cell lysates and cell supernatant containing PV. Bovine serum albumin (BSA) (Bio-Rad) was used as the standard following manufacturer's instructions and diluted in PBS along with the samples in duplicates. Protein concentrations of samples were unknown hence two standards were prepared in parallel with different ranges of protein concentration each requiring their own specific volume input. Standard-A had a range of 125-2000 µg/ml BSA and 5 µl of standard or sample were mixed with 250 µl of 1x dye reagent per well in a 96-well plate. Standard-B 1.25- 25 µg/ml 150 µl of standard or sample were mixed with 150 µl of 1x dye reagent per well. Samples were incubated for 5 min at R.T and the absorbance was read at 595 nm using a spectrophotometer.

2.5.3 Western Blots and sample preparation

In order to confirm protein expression Western Blot analysis was used to visualise EBOV protein production including sGP and EBOV GP with commercially produced anti-EBOV Abs or naturally produced Abs present in CP of Ebola survivors. For the sGP research, two cell culture media were used to produce sGP. Transfection was carried out as described (2.3.3) however 24 h post-transfection cell culture medium was replaced with either DMEM containing no FBS or SFM (Gibco). Supernatant was harvested 72 h post-transfection and protein was concentrated as described (2.5.1). Commercially produced, purified sGP (2BScientific) was also utilised in a western blot. Samples for SDS-PAGE were prepared as described below (Table 2.1) (Taken from Invitrogen quick reference).

Table 2.1 Reduced and non-reduced sample preparation for SDS-PAGE

Reagent	Reduced Sample	Non-reduced sample
Sample (10 µg/ well)	X µl	X µl
Tris-Glycine SDS Sample Buffer (2x)	20 µl	20 µl
NuPAGE™ Reducing Agent (10x)	4 µl	-
Deionised water	To total volume	To total volume
Total Volume	40 µl	40 µl

Samples were heated at 85°C for 2 minutes and loaded promptly onto an SDS-PAGE gel.

2.5.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated on SDS polyacrylamide (PAGE) gels based on their molecular size using 4-12% Bis-Tris gradient, mini wedge, 1 mm, 12-well gels (Invitrogen) using a mini gel tank (Invitrogen). Each chamber was filled with 400 ml of 1x Novex™ Tris-Glycine SDS Running buffer. Combs were removed from gels and placed with clamps in the tank ready for sample loading into the wells of the gel. A maximum of 40 µl sample was loaded into each well. The gel was run at 100 V for 1h 30 to allow protein migration through the polyacrylamide matrix.

2.5.5 Protein transfer

Proteins separated on the basis of molecular size by SDS-PAGE were then transferred by the iBlot™ 2 gel transfer device to a membrane prior to immuno-blotting. Instructions were followed as per manufacturer's protocol, in brief the pre-run gel was removed from its cassette and the wells were removed. A transfer stack containing a nitrocellulose membrane was placed onto the iBlot™ device, the gel was briefly immersed in ddH_2O and placed onto the membrane. An iBlot™ filter paper pre-soaked in ddH_2O was placed on top of the gel, the transfer gel/ copper electrode (top stack) was positioned over the filter paper and an iBlot™ 2 Absorbent Pad containing electrical contacts was placed on top. A blotting roller was used to remove air bubbles between each layer added to the stack. The transfer programme used was p0, 7.5 min 25 V.

2.5.6 Immuno-blotting

Proteins were visualised using the iBind™ Western System and instructions were followed as per manufacturer's protocol. Briefly, the blotted membrane (protein-side up) was immersed in 5 ml of 1x iBind™ solution (used for blocking, diluting Abs, washing and wetting the iBind™ Card). The iBind™ Card was placed into the iBind™ Western Device, 5 ml of 1x iBind™ solution was pipetted onto the card with another 1 ml pipetted to pool in the centre of the card. The membrane was placed protein-side down on the centre of the card, air bubbles were removed with the blotting roller and the lid was closed.

Solutions were added sequentially as described below (Table 2.2):

Table 2.2 Immunoblot reagent volumes using iBind

Well	Solution
1	2 ml Primary Antibody (2 ml, diluted with 1x iBind™ solution at the manufacturers recommended dilution)
2	2 ml of 1x iBind™ solution
3	2 ml Primary Antibody (2 ml, diluted with 1x iBind™ solution at 5x the manufacturers recommended dilution)
4	6 ml of 1x iBind™ solution

Primary Abs used in EBOV detection include anti-EBOV GP Abs FE25 (Enzo), FE37 (Enzo), FE18 (Thermo Fisher), EBOV survivors CP (Donor 45 and 49). Secondary Abs include Goat anti-mouse HRP (Santa-Cruz Biotechnology) and Goat-anti human (Abcam). Incubation was approximately 2.5 h. The membrane was washed twice in 20 ml of ddH_2O prior to the detection protocol.

2.5.7 Visualising proteins

The Pierce™ ECL Western Blotting Substrate is a chemiluminescent substrate for the detection of HRP on immunoblots. Detection reagents 1 and 2 are mixed in equal parts and 0.125 ml of working solution is used per cm^2 of the blot. The blot is incubated for 1 min at R.T. with the working solution prior to being placed in a clear plastic sheet. Proteins were then visualised using the ChemidocX exposure time 10 min, 30 s exposure.

2.6 Mathematical modelling

The mathematical modelling was conducted by Dr R. Sharma, Liverpool School of Tropical Medicine (LSTM). Exponential functions were fitted to individual longitudinal data for the PV neutralisation, DABA and the RCE PRNT assays to obtain respective Ab endpoints. The function fitted was the typical analytic form for first order growth/decline:
Ab level= Ae^(kt)

A is a fitted pre-exponential term, k is the first order rate constant of growth or decline and t is time post convalescence in days.

Growth and decline Ab data were then fitted separately and median and variance values for growth and decline were calculated and compared for each end point by:
Doubling time/ Half-life = ln(2)/k

Individuals for which there was greater than or equal to three longitudinal data points were included in the analysis, in order ensure adequate characterisation of the curvature of decline/growth."

2.7 Statistical Analysis

Statistical analyses of data were implemented using GraphPad Prism 6.0 software. Unpaired sample comparisons were conducted for all data however individual figures state the corresponding statistical test performed. These include:

- Parametric and non-parametric t-tests (student t-test and Mann-Whitney U test)
- Parametric and non-parametric ANOVA (Ordinary ANOVA and Kruskal-Wallis test)
- Linear regression analysis
- Correlation coefficient

P values were depicted by *: * P value< 0.05, ** P value< 0.01, *** P value< 0.001, **** P value< 0.0001.

3 Establishing and optimising an Ebola pseudo-typed virus assay

3.1 Introduction

3.1.1 Replication competent EBOV

The highly infectious nature of viral haemorrhagic fevers such as EBOV, are pathogens perceived to present the most significant health risks to humans and/ or animals and are therefore categorised as a BSL-4 pathogen (Janosko et al., 2016). Accordingly, research in BSL-4 requires adherence to rigorous lab practices and standard operating procedures for entering and exiting a BSL-4 containment lab including the use of chemical showers upon exit, biohazard suits need to be worn within the lab and highly specialised and robust laboratory facilities need to be in place (Janosko et al., 2016). In addition, depending on the governmental law of the country, further assessments may need to be undertaken. EBOV is deemed a Tier 1 select agent and is therefore categorised as a high-consequence, bio-terrorism agent. Staff intending to work with these pathogens must undergo a security risk assessment with the government prior to commencing work. Also for those working with Tier 1 select agents, staff may be enrolled in a programme which continuously monitors and evaluates the physical and mental health of researchers ensuring they are able to work in a safe manner (Janosko et al., 2016).

The 'gold standard' serological assays for infections are considered virus neutralisations with replication competent viruses; however, the strict biosafety requirements needed to culture EBOV, limits the studies that can be carried out (Sarzotti-Kelsoe et al., 2014). The stringent lab practices means there are limitations on experimental techniques; experiments take a longer duration as factors such as entering and exiting the lab need to be taken into consideration (Janosko et al., 2016).

3.1.2 Pseudo-typed EBOV

To address the above issues an alternative approach is to use PV in place of replication competent viruses. PV are recombinant viral particles composed of a
backbone and envelope (Env) proteins derived from different viruses. The backbone has been modified to prevent expression of the *env* gene hence requiring an additional plasmid to express the surface proteins required to produce the desired PV (Sanders, 2002). PV are unable to continually replicate and are capable of only one round of infection in a host cell line, permitting them to be safely handled in a biosafety level 2 (BSL-2) laboratory with manageable lab practices thus allowing a more extensive range of experiments to be carried out (Li et al., 2018). The single round infection capacity of these viruses allows for a more reliable analysis of compounds aimed at blocking viral entry compared to viruses which undergo multiple rounds of replication.

The GP sequence of the virus of interest is used for the production of PV. Hence the exterior proteins of the PV bears high similarity to the native viral proteins, which can be used to mediate viral entry efficiently into host cells (Li et al., 2018). For this reason, PV have been greatly utilised in studies of cellular tropism and receptor recognition (Bartosch et al., 2003), the inhibition of viruses (J. Wang et al., 2014) as well as developing and characterising Abs and vaccines (Robinson et al., 2016). Further support for the PV system comes from data showing good correlation between *in vitro* anti-viral assays and *in vivo* bio-distribution analyses carried out with replication competent viruses (Wright et al., 2008).

PV can encode reporter genes, usually an enzyme utilised in chemiluminescence such as luciferase, or a fluorescent protein such as green fluorescent protein (GFP). Each reporter has their respective benefits and weaknesses. Assays utilising chemiluminescence are more sensitive with lower background, however, experiments can be costly. Whilst experiments with fluorescent proteins are cheaper and easier to use in both *in vitro* and *in vivo* systems, detection may be less sensitive with a possibility of higher background interference. PV infection of host cell lines has been demonstrated to be directly proportional to the expression of the reporter gene thus permitting easy quantitation analysis of the PV (Liu et al., 2012).

3.1.3 Aims of this chapter

Various parameters affecting the production, infectivity and luciferase detection of EBOV PV were investigated in a systematic process to establish the relevant optimal conditions.

These included:

1. Transfection period
2. Infectivity of EBOV into different cell lines
3. The effect of dextran on virus infectivity
4. Fresh vs frozen virus on infectivity
5. Titration of EBOV envelope during transfection
6. Intra vs Inter EBOV virus infectivity
7. Promega vs Thermo Fisher luciferase detection kits
8. Different PEI batches on virus production
9. Cell passage number on virus infectivity

Following the determination of the optimal conditions for PV production and infection, these stocks were used to evaluate the ideal conditions for the EBOV PV neutralisation assay. Including assessing:

1. EBOV negative human plasma
2. NIBSC standard plasma for PV neutralisation assay
3. EBOV CP
4. Different batches of PV on neutralisation titres
5. Plate layout on virus infection

3.2 Results

3.2.1 Generation and characterisation of EBOV GP HIV-1 pseudo-typed virus

The first step was to evaluate the key conditions influencing EBOV PV production and infectivity.

3.2.1.1 EBOV strains, description and rationale

Sequenced EBOV GP strains from the 1995 and 2013-2016 epidemic were utilised in the design of plasmid constructs for the subsequent production of EBOV PV. The descriptions and corresponding EBOV IDs of the plasmid constructs used in this thesis are described in (Table 3.1) and the EBOV IDs are used to refer to the virus strain thereafter.

Table 3.1 EBOV strains and corresponding IDs

A description of the EBOV strains and variants used in this study as well as the corresponding IDs.

EBOV ID	Description of EBOV strains
M-14	Makona- 2014 M-14 is a variant of the Zaire Makona strain and is identical to the Makona C5 strain; one of the three earliest EBOV isolates to be sequenced in the 2013-2016 epidemic commencing in Guinea (Baize et al., 2014).
MEM-14	Makona Early Mutations- 2014 Based on the M-14 sequence, MEM-14 includes mutations that were seen repeatedly in the field, early in the epidemic. These mutations in the GP are illustrated in the BioEdit alignment (Fig 3.1). A letter indicates an amino acid that is different to the consensus sequence M-14 at that position.
K-95	Kikwit- 1995 Sequenced isolate from the 1995 outbreak in Kikwit, Democratic republic of Congo (DRC). This strain was also utilised in the rVSVΔG-ZEBOV-GP ring vaccination during the epidemic (Heppner et al., 2017).
L-14	Lomela- Lokolia- 2014 Sequenced isolate from the 2014 outbreak in DRC, unrelated to the 2013-2016 epidemic (Maganga et al., 2014).
MSEM-14	Makona Single Early Mutations-2014 Based on the M-14 sequence, MSEM-14 includes single GP mutations that were observed only once in the epidemic. These mutations are illustrated in the BioEdit alignment (Fig 3.4), a letter indicates an amino acid that is different to the consensus sequence M-14 at that position.

EBOV M-14 (Makona C5) was utilised for experiments as it was one of the three earliest (Makona C5, C7 and C15), sequenced strains isolated from patients in Guinea in the 2013-2016 epidemic (Baize et al., 2014)c. Makona C5, C7 and C15 sequences were identical with the exceptions of a few polymorphisms (Baize et al., 2014). K-95 is one of the major outbreak variants of the Zaire ebolavirus strain and was also utilised as the EBOV antigen in the V920 vaccine implemented during the 2013-2016 epidemic (Heppner et al., 2017). The L-14 EBOV strain was responsible for a concurrent EBOV outbreak in the DRC as to the 2013-2016 epidemic. Clinical and epidemiological characteristics of L-14 were similar to those of previous EVD outbreaks in equatorial Africa however, the causative agent was a local variant and had zoonotic origins that were different from that in the 2013 epidemic in West Africa and was deemed to be more closely related to the 1995 Kikwit variant (Maganga et al., 2014). By studying the capacity of the 2014 Makona CP to neutralise variants of Zaire ebolavirus; K-95 and strains quite distinct from Zaire; L-14, deductions can be made as to the cross-protection of CP. EBOV M-14 containing mutations seen repeatedly; MEM-14, and singly; MSEM-14, in the epidemic were studied with the idea that any observed differences in neutralisation would be further scrutinised by studying the individual mutations incorporated into the M-14 strain.

3.2.1.1.1 EBOV amino acid sequence analysis

The EBOV GP is the only exposed viral protein and is essential for virus entry into target cells (White and Schornberg, 2012). In this study PV was produced with the GP of five strains of EBOV, the GP amino acid sequences of each strain are depicted (Figure 3.1). BioEdit was used to perform a simple GP amino acid alignment between the five EBOV strains with M-14 used as a reference. Dots indicate the sequence is the same whilst a letter indicates that an amino acid at that specific location is different.

The previously discussed (1.12.1.2 and Table 1.4) mapped functional mAb epitopes of the EBOV GP found to be protective against the Zaire EBOV strain, (Ponomarenko et al., 2014) are also highlighted in Figure 3.1. Examination of the EBOV GP amino acid sequences indicated that there were no prominent differences between the EBOV strains in the epitopes that are targeted by the protective mAbs (Figure 3.1). Therefore, it could be inferred that CP NAbs will not exhibit differential neutralisation potential between the EBOV PV strains. Conversely studies looking into the functional significance of amino acid substitutions in EBOV sequences during the outbreak highlighted prominent amino acid mutations; A82V

and T554I (Dietzel et al., 2017; Ruedas et al., 2017) in the EBOV GP. Mutations affecting GP function can alter virus growth properties (Ruedas et al., 2017). EBOV with the Alanine (A) - Valine (V) amino acid substitution in the RBD of GP at position 82 appeared early in the outbreak and became fixed in the population. This mutation was shown to increase, viral infectivity and increase tropism in human primary and continuous cell lines in cell based assays, supporting the hypothesis that A82V was a fitness adaptation (William E. Diehl et al., 2016; Urbanowicz et al., 2016b). Position 544 has been suggested to play a significant role in pathogenesis, with publications indicating some sequences code for threonine (T) whilst others code for isoleucine (I) at this position. Several explanations exist for this amino acid substitution, however, one study found the spontaneous T554I mutation in EBOV GP was sufficient to confer tissue culture adaptation in Zaire ebolavirus that lead to enhanced viral entry compared to wild type virus strains (Ruedas et al., 2017). The MEM-14 EBOV strain is the only strain with the V amino acid mutation and both the MEM-14 and M-14 EBOV strains have the I adaptation (figure 3.4) however, the amino acid mutations at these points were not observed to confer enhanced infectivity of the respective EBOV strains.

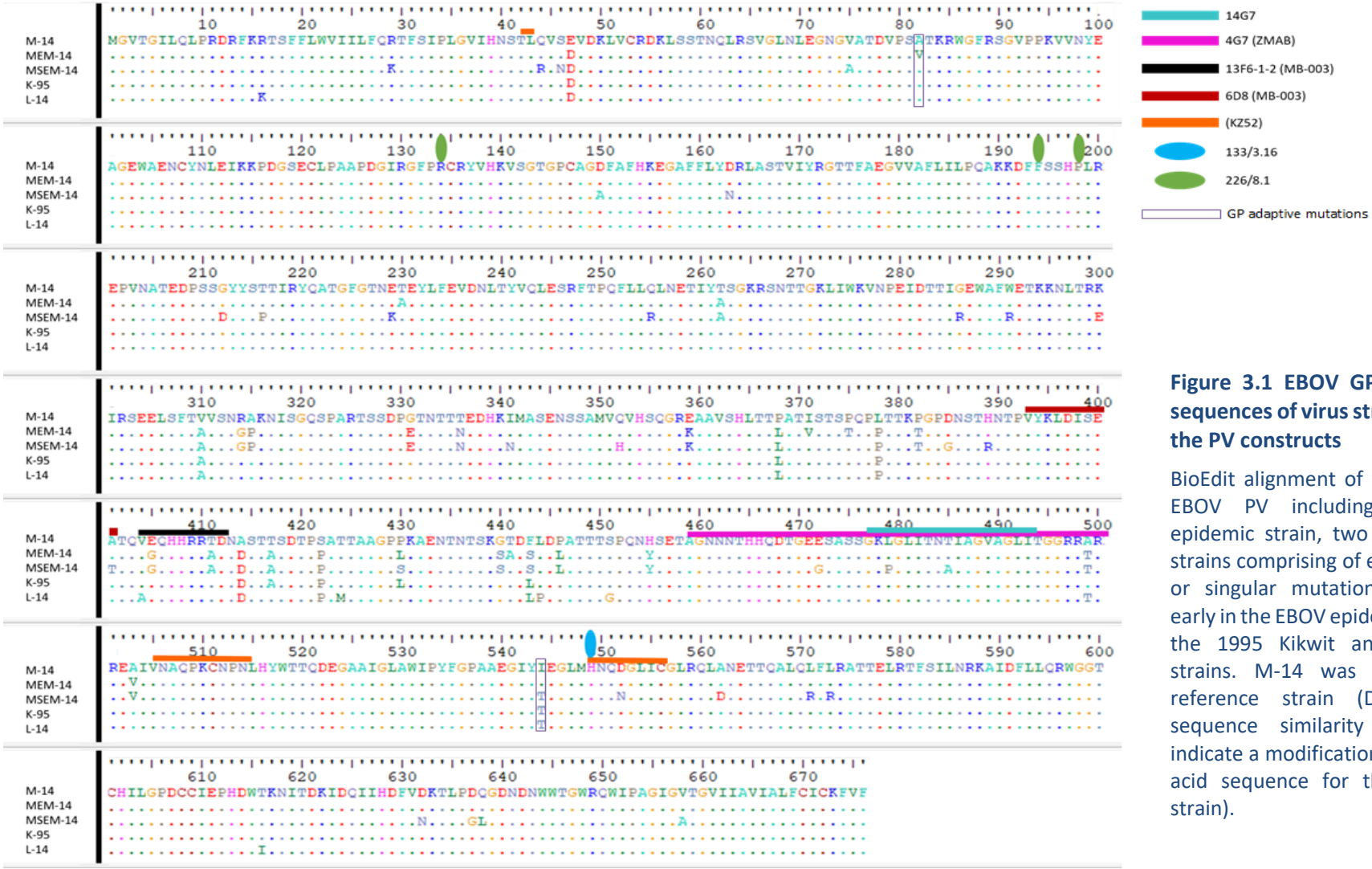


Figure 3.1 EBOV GP amino acid sequences of virus strains used for the PV constructs

BioEdit alignment of five strains of EBOV PV including the; 2013 epidemic strain, two 2013 mutant strains comprising of either multiple or singular mutations that arose early in the EBOV epidemic as well as the 1995 Kikwit and 2014 DRC strains. M-14 was used as the reference strain (Dots indicate sequence similarity and letters indicate a modification in the amino acid sequence for that particular strain).

3.2.1.2 pNL4-3.Luc.R-E EBOV GP virus characterisation

3.2.1.2.1 Effect of 48 h vs 72 h transfection on EBOV infectivity

HEK293T cells were the primary choice for use in the transfection procedure as they are a well-established transient expression system. The characteristics that make the cell line desirable for use include; fast reproduction and minimal maintenance, a high volume of cells per flask and highly efficient transfection and protein production. As HEK293T are a mammalian cell line, they allow post-translational modifications such as glycosylation of produced proteins, which are essential for the production of the EBOV GP and subsequently virus entry into host cell lines. In order to assess which period; 48 or 72 h was optimal for virus production pNL4-3.Luc.R-E virus stocks were produced, including the negative control ΔEnv, the positive control bearing the VSVg GP and EBOV GP MEM-14. Each of the virus stocks were harvested at either 48 h or 72 h post-transfection and tested for infectivity efficiency. The transfection period did not influence luciferase activity of VSVg stocks, where in both instances the upper limit of detection was reached. However virus stocks harvested at 72 h transfection had significantly higher EBOV infectivity than virus harvested at 48 h post transfection ($p < 0.001$) (Figure 3.2). To maintain consistency for further experiments, 72 h post transfection was adopted for harvesting virus stocks.

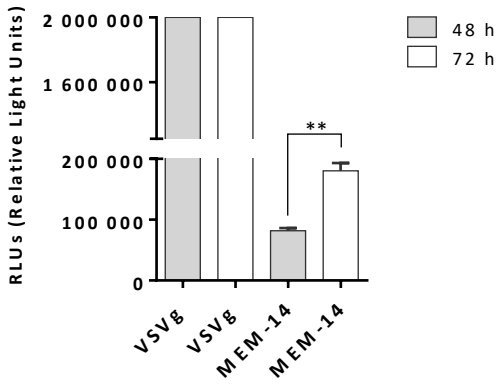


Figure 3.2 EBOV Infectivity of virus harvested 48 h vs 72 h post-transfection

Infection of pNL4-3.Luc.R-E VSVg and MEM-14 virus stocks harvested at either 48 (grey) or 72 h (white) post-transfection. T-test: Two-sample assuming equal variance was used to analyse significance. A statistical difference was concluded between virus harvested at 48 h and 72 h for MEM-14 stocks (**$p<0.01$). Technical repeats (n= 4) and biological repeats (n= 2) were conducted. Error bars represent mean with standard deviation.

3.2.1.2.2 Comparison of infectivity of pNL4-3.Luc.R-E EBOV GP in different cell lines

A comparative study was conducted to investigate whether virus stocks bearing GP of the previous, recent and modified EBOV strains, harvested at 72 h post-transfection were able to infect the same range of cell lines. The cell lines included four mammalian cell lines, three human; HEK293T (Human embryonic kidney cells), TZM-bl (HeLa cell derivative) and Huh7 (Hepatocyte cells) as well as a monkey mammalian cell line, Vero (African green monkey cells). pNL4-3.Luc.R-E was co-transfected respectively with all previously described EBOV GP strains, including those originating from DRC, Guinea and the modified GPs that were produced in order to study potential immune escape of EBOV.

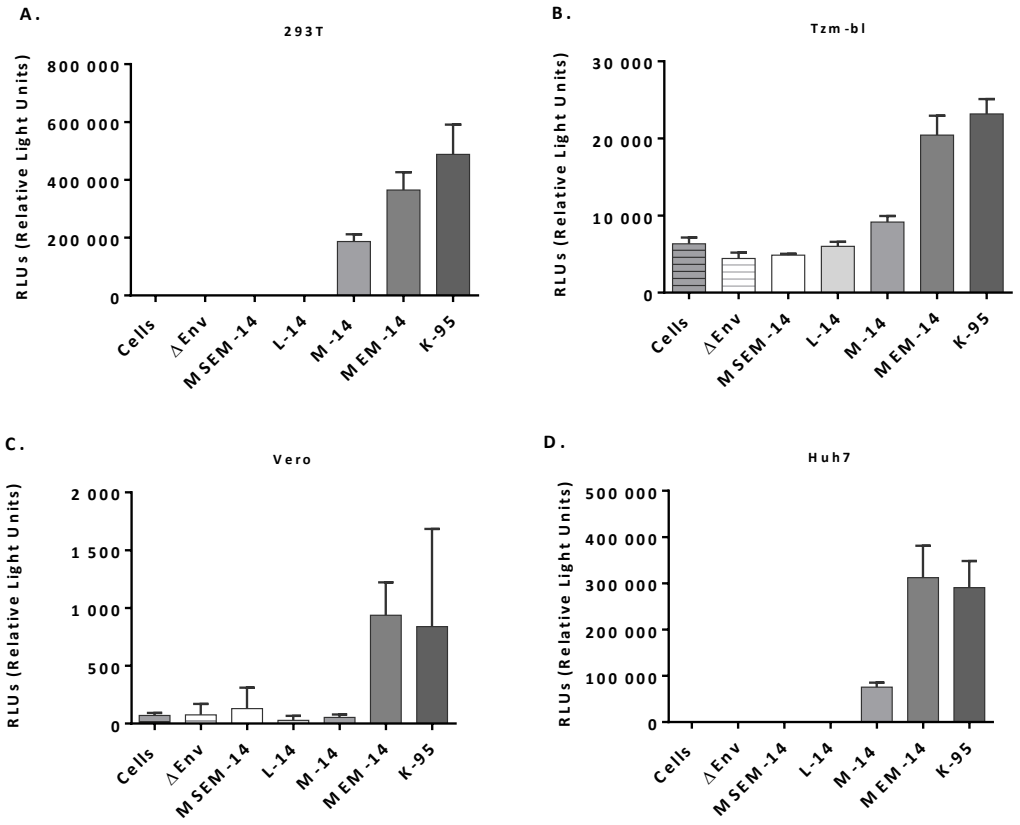


Figure 3.3 Infectivity of different EBOV strains into various cell lines

Detection of differential luciferase activity in cell lines A) 293T, B) TZM-bl, C) Vero and D) Huh7, infected with pNL4.3.Luc.R-E EBOV strains; MSEM-14, L-14, M-14, MEM-14 and K-95. EBOV infectivity were compared to the luciferase activity of negative controls; cells and backbone alone. Technical repeats (n= 3) and biological repeats (n= 2) were conducted for this experiment. Error bars represent mean with standard deviation.

From the comparative analysis, it was observed that overall; cell lines HEK293T and Huh7 cells yielded the highest infectivity. Vero cell lines have been previously utilised extensively for testing the infectivity of EBOV PV, surprisingly however, luciferase activity was not detected in Vero cells suggesting they could not be infected with any of the EBOV PV stocks. TZM-bl cells contain a luciferase reporter and produce background levels of approximately 3000-7000 Relative light units (RLUs). EBOV infectivity was overall not much greater than the background levels of TZM-bl cells. Unexpectedly, it was also observed that pNL4-3.Luc.R-E EBOV strains L-14 and MSEM-14 were repeatedly unable to infect any of the cell lines (Figure 3.3).

3.2.1.2.3 Effect of dextran on EBOV infectivity

Previous data in the field of HIV-1 research has shown that infection of HIV-1 PV in the presence of the polycation, Diethylaminoethyl (DEAE)- Dextran increased the levels of PV infection (Sarzotti-Kelsoe et al., 2014). To see whether the carbohydrate polymer could aid in the infectivity of EBOV stocks previously shown to have background or no detectable luciferase activity, virus supplemented with DEAE-Dextran was used to infect HEK293T, TZM-bl and Huh7 cells alongside the positive control VSVg (Figure 3.4).

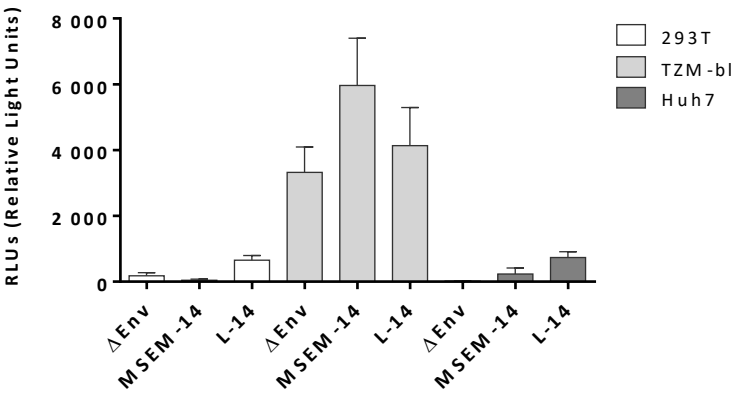


Figure 3.4 Infectivity of MSEM-14 and L-14 in the presence of dextran

EBOV strains pNL4.3.Luc.R-E MSEM-14 and L-14 were incubated with the polycation, dextran prior to infection into HEK293T (white), TZM-bl (light grey) and Huh7 (dark grey) cell lines. EBOV PV infectivity was compared to infectivity of the negative control; backbone alone within each respective cell line. Technical repeats (n= 3) and biological repeats (n= 2) were conducted for this experiment. Error bars represent mean with standard deviation.

It was observed that dextran did not improve the infectivity of EBOV strains MSEM-14 and L-14 virus stocks. Infectivity remained at background levels for the respective cell lines (Figure 3.4) apart from VSVg; which reached luciferase detection levels at the point of saturation (data not shown). EBOV GP K-95 previously shown to have good infectivity in HEK293T and Huh7 cells (Figure 3.3 A +D) was also infected onto the above mentioned cell lines in the presence of DEAE- Dextran (data not shown), however no increase in infectivity was observed. There was no increase in infectivity with the use of Dextran therefore it was not used further in infectivity experiments.

3.2.1.2.4 The effect of freezing on infectivity

Thus far the experiments conducted to investigate parameters affecting pNL4-3.Luc.R-E virus infectivity were carried out with virus stored at 4°C post-harvesting. A key factor considered when virus infectivity was characterised was the effect a freeze-thaw cycle could have on the virus. The ability to freeze-thaw a virus stock without substantial loss of infectivity meant a large batch of virus could be produced, pooled and frozen at one time, thus allowing a set of experiments to be carried out with one batch, making results more comparable. pNL4-3.Luc.R-E was produced and aliquots were stored at either 4°C or -80°C for 24 h prior to infection into Huh7 cells. Luciferase activity of the infected cells were measured revealing that EBOV strains M-14, MEM-14 and K-95 had been rendered inactive by storage at -80°C and had completely lost the ability to infect the host cell line (Figure 3.5). Therefore, pNL4-3.Luc.R-E virus stocks would not be able to be stored at -80°C for long-term use.

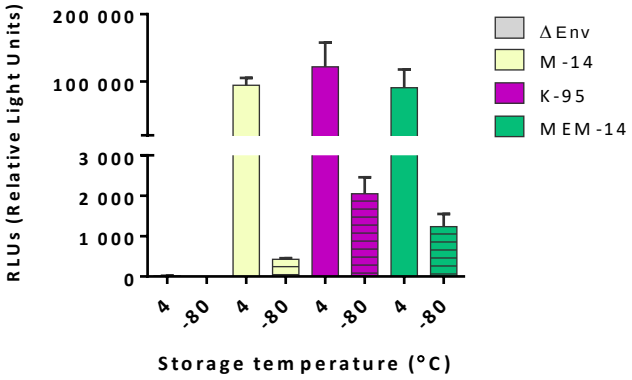


Figure 3.5 Infectivity of pNL4-3.Luc.R-E EBOV virus stocks stored at 4°C and -80°C prior to infection

Post-harvesting, aliquots of pNL4-3.Luc.R-E M-14 (yellow), K-95 (purple), and MEM-14 (green) virus stocks were stored at either 4°C or -80°C prior to infection into Huh7 cells. Luciferase activity was determined 48 h post-infection, error bars represent mean with standard deviation. Technical repeats (n= 3) and biological repeats (n= 2) were conducted.

Based on these results an alternative was to produce EBOV virus stocks with pSG3$^{\Delta env}$, a HIV-1 retroviral backbone. However, as pSG3$^{\Delta env}$ does not have a reporter gene in the backbone the resulting virus produced, would need to be infected into a reporter cell line such as TZM-bl expressing luciferase to be able to measure infectivity. TZM-bl cells were infected with pSG3$^{\Delta env}$ virus stocks that had either been stored at 4°C or -80°C for 24 h prior to infection.

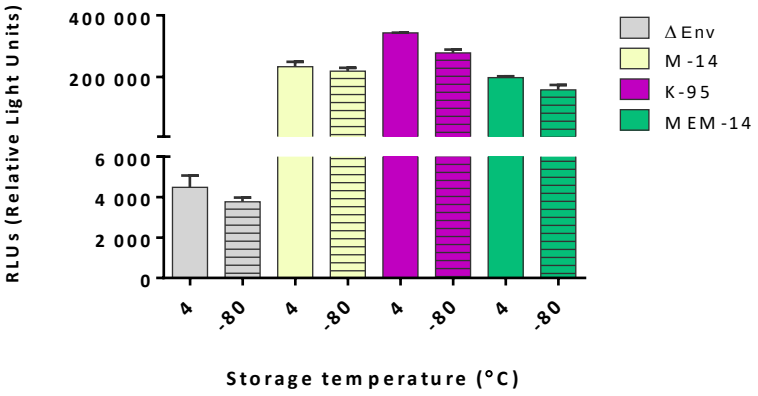


Figure 3.6 Infectivity of pSG3$^{\Delta env}$ EBOV virus stocks virus stocks stored at 4°C and -80°C prior to infection into TZM-bl cells

Post-harvesting, aliquots of pSG3$^{\Delta env}$ M-14 (yellow), K-95 (purple), and MEM-14 (green) virus stocks were stored at either 4°C or -80°C prior to infection into TZM-bl cells. Luciferase activity was determined 48 h post-infection and compared to infectivity of the negative control, backbone alone (grey) with error bars representing mean with standard deviation. This experiment was reproduced technically (n= 4) and biologically (n= 2).

The results showed that pSG3$^{\Delta env}$ EBOV stocks could withstand a freeze-thaw cycle. Up to 40% decrease in virus infectivity was observed overall, however, frozen virus aliquots retained their infectivity (Figure 3.6). Therefore, large batches of virus stocks could be produced and frozen for use in multiple experiments. Conclusions drawn from previous experiments such as the period of transfection and omitting the use of dextran were retained for simplicity.

3.2.1.3 Characterisation of pSG3 EBOV GP pseudo-typed virus

Further experiments were conducted with the pSG3$^{\Delta env}$ EBOV PV to determine the ideal conditions for production, in order to understand variation that could be expected between different batch productions and to investigate if infectious virus could be produced with pSG3$^{\Delta env}$ and EBOV strains MSEM-14 and L-14.

3.2.1.3.1 Titration of EBOV GP Env plasmid during transfection

To optimise the production of pSG3$^{\Delta env}$ EBOV virus stocks, a parameter investigated was the effect of Env input during transfection and its subsequent effect on infectivity. Different amounts of EBOV Env plasmid ranging from 855 ng to 47.5 ng of EBOV strain M-14 were used for transfection alongside 2000 ng of pSG3$^{\Delta env}$ plasmid, respectively. Produced virus were harvested 72 h post-transfection, aliquoted and frozen at -80°C prior to infection into TZM-bl cells, frozen aliquots were also used in a p24 ELISA. It was observed that all Env input amounts produced infectious stock, however, higher Env input resulted in low EBOV infectivity seen with 855 ng input. Infectivity of the virus increased with a reduction in Env input up until approximately 285 ng Env input, where virus infectivity was observed to be comparable (Figure 3.7).

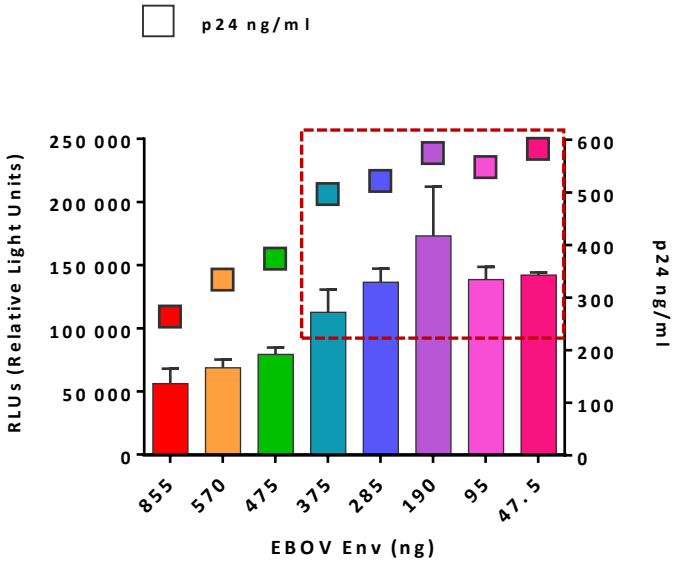


Figure 3.7 Titration of EBOV Env input during transfection and corresponding p24 quantitation of resulting virus

EBOV strain M-14, Env amounts 855- 47.5 ng were titrated alongside 2000 ng of backbone during the transfection process. The resulting virus was measured for infectivity in TZM-bl cells (bars) and quantified by a p24 capsid ELISA (squares). Corresponding virus stock infectivity and p24 titres are indicated by the same colour and error bars represent mean with standard deviation. The experiment was reproduced technically (n= 3) and biologically (n= 3).

The virus infectivity profile was mirrored in the quantitation of the p24 capsid protein; higher Env inputs equated to lower p24 concentration; a measure of virus backbone production. These results mirror other studies which shows that the recovery of infectious particles was

greatly affected by the amount of GP construct and packaging vector plasmids used to create PV (Mohan et al., 2015; Urbanowicz et al., 2016a). A range was identified where varied amounts of plasmid input yielded similar virus infectivity, which was also reflected in the p24 production of the virus stocks. Based on this data as well as through consultation with a research group who had extensively utilised a similar EBOV PV system; 285 ng EBOV Env was elected for all ensuing transfections to produce EBOV PV.

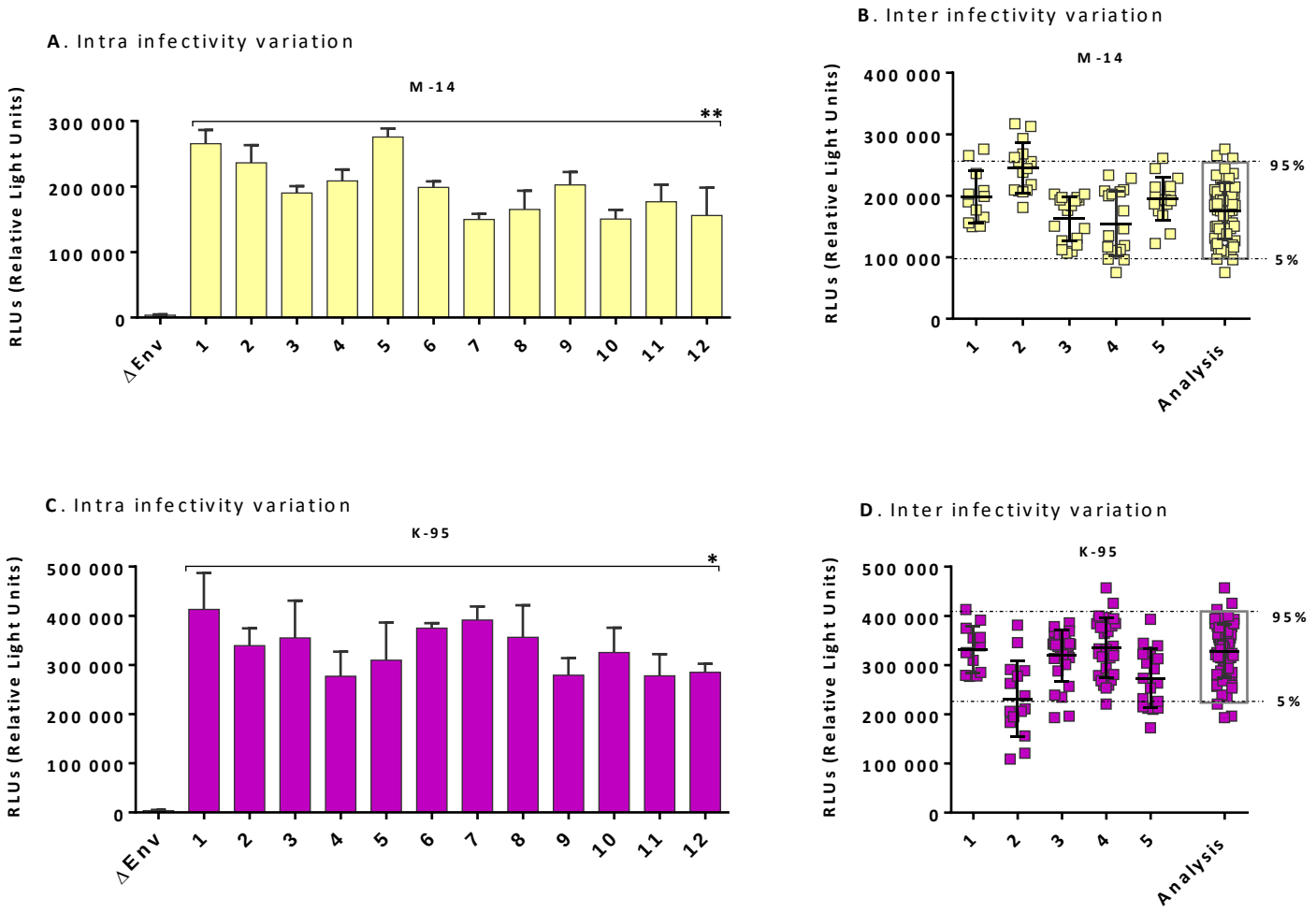
3.2.1.3.2 Intra and Inter infectivity variation between virus batches

The PV system is complex and where many factors can affect virus production and consequently virus infectivity. A procedure was developed to try and ensure frozen virus stocks had the same range of infectivity between different batches of produced virus for a given EBOV strain. Firstly to assess the variation in infectivity each EBOV strain, PV harvested from individually transfected dishes were tested for infectivity; aliquots of the PV were frozen at -80°C prior to infection of TZM-bl cells.

The infectivity of PV produced in each batch for the respective EBOV strain is shown in A) M-14, C) K-95 and E) MEM-14 (Figure 3.8) illustrates differences in infectivity between dishes transfected concurrently for respective viruses. Statistical difference between infectivity of the harvested PV for each respective strain was analysed with the Kruskal-Wallis test. No significant differences were observed for C) K-95 and E) MEM-14 (Figure 3.8), however, a statistically significant difference (P= 0.05) was observed for infectivity of A) M-14 PV. Following on from this it was deemed important to assess the variation in infectivity that could be expected between different batches produced for the same EBOV strain. Batches of EBOV PV were produced on four more occasions, as described before where aliquots of the harvested PV were frozen at -80°C prior to infection of TZM-bl cells.

The infectivity of PV produced in each batch for each EBOV strain is shown in B) M-14, D) K-95 and F) MEM-14 (Figure 3.8). Variation in infectivity between batches were analysed with the Kruskal-Wallis test. The batches observed to influence significant differences; B) batch 2 D) batch 2 and 5 and F) batch 3 were excluded in the next step of analysis. In order to statistically establish an acceptable range of virus infectivity for each virus strain, RLUs within the remaining batches were collectively analysed (labelled analysis on graphs) with column statistics; 95% and 5% percentile (grey box) in order to exclude outliers.

To ensure virus stocks with similar levels of infectivity for respective strains would be utilised in ensuing experiments an exclusion criteria; a pre-determined infectivity range was put into place for each virus strain (black dotted lines). The RLUs of produced virus which did not fall within the pre-determined range for the respective virus strains were discarded. Differences in infectivity between batches of produced virus could be expected and the exclusion criteria allowed for the production of virus stocks with similar levels of infectivity for the respective viruses.


A. Intra infectivity variation
M-14
RLUs (Relative Light Units)
300 000
200 000
100 000
0
**
ΔEnv
1
2
3
4
5
6
7
8
9
10
11
12
B. Inter infectivity variation
M-14
RLUs (Relative Light Units)
400 000
300 000
200 000
100 000
0
95%
5%
1
2
3
4
5
Analysis
C. Intra infectivity variation
K-95
RLUs (Relative Light Units)
500 000
400 000
300 000
200 000
100 000
0
*
ΔEnv
1
2
3
4
5
6
7
8
9
10
11
12
D. Inter infectivity variation
K-95
RLUs (Relative Light Units)
500 000
400 000
300 000
200 000
100 000
0
95%
5%
1
2
3
4
5
Analysis

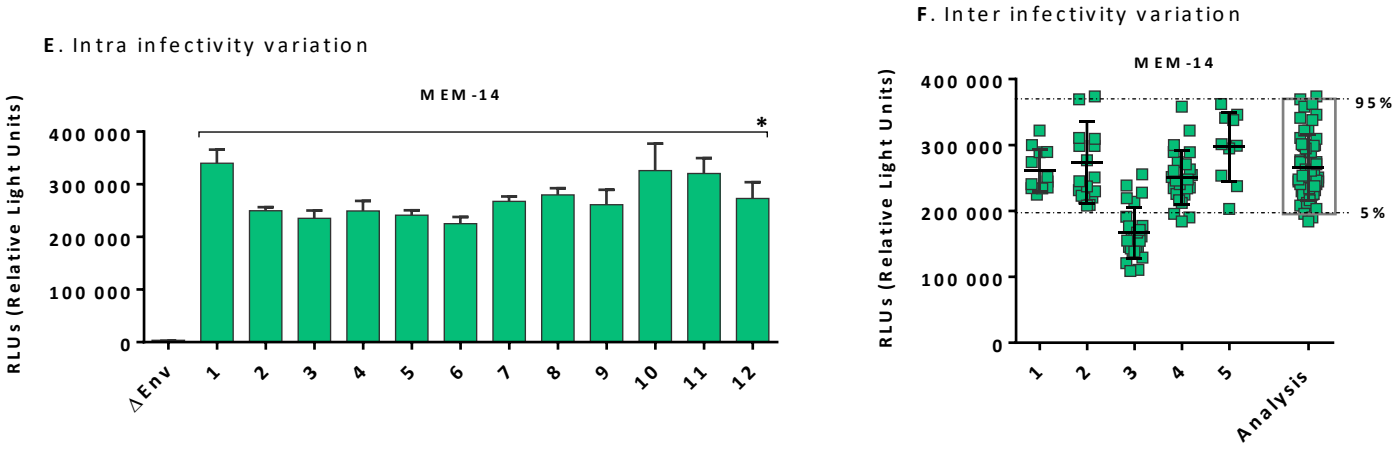


Figure 3.8 Intra and Inter infectivity variation of different EBOV strains

The infectivity of PV harvested from individually transfected 10 cm dishes were statistically assessed for differences with the Kruskal-Wallis test. Significant differences were observed for A) M-14 (**p<0.01), C) K-95 and E) MEM-14 (*p<0.05). Based on this result additional batches of EBOV PV B), D) and F) were produced to statistically assess variation in infectivity. Batches that did not influence statistical significance (shown in analysis column) were used to compute the 95%, 5% percentile (grey-dash box). A PV infectivity exclusion criteria for the respective viruses was used to evaluate newly produced batches of PV (shown as dotted lines). Error bars represent mean with standard deviation.

3.2.1.3.3 PV production of pSG3 with EBOV MSEM-14 and L-14

To investigate the possibility of EBOV being able to evade the immune system due to random mutations in the Env GP, a plasmid; MSEM-14 was designed with mutations that arose early in the Ebola epidemic. Subsequent virus stocks produced would have these mutations in the envelope GP. As well as the outbreak, which caused the epidemic in West Africa, there was a smaller unrelated outbreak in DRC in July 2014. A plasmid was produced with the sequence of this strain; L-14. Both plasmids were transfected alongside the $pSG3^{\Delta env}$ and produced virus was subsequently tested for infectivity of TZM-bl cells as previously described.

MSEM-14 and L-14 demonstrated very low infectivity as compared to the three previously described EBOV PV produced (Figure 3.9 A and B). Both plasmids were utilised for transfection in HEK293T cells multiple times and tested for infectivity in TZM-bl cells, which again resulted in the same range of low RLUs.

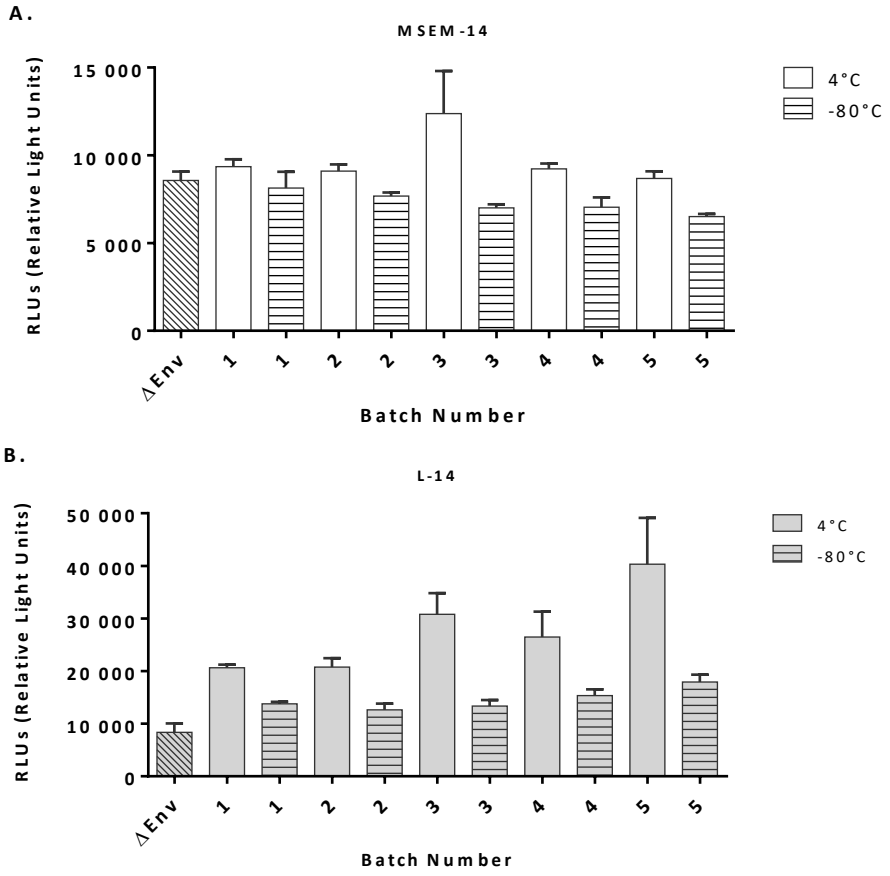


Figure 3.9 Infectivity of EBOV strains MSEM-14 and L- 14

EBOV strain A) MSEM-14 and B) L-14 were co-transfected respectively alongside pSG3$^{\Delta env}$ into 5 transfection dishes seeded with 293T cells. Aliquots of virus stocks were stored at 4°C or -80°C prior to infection into TZM-bl cells. Luciferase activity was measured 48 h post-infection and compared to the negative control, infectivity of backbone alone. Error bars represent mean with standard deviation.

To consider whether Env GP plasmid amounts had an effect on virus production and the resulting infectivity, L-14 was titrated during transfection. It was seen that Env GP titration did not have an effect on infectivity of L-14 (Figure 3.10).

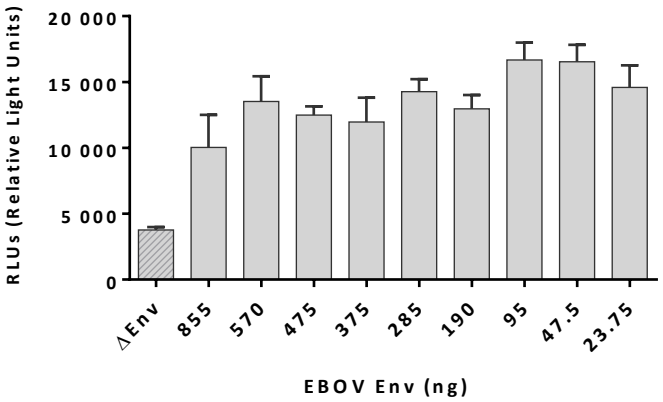


Figure 3.10 Titration of EBOV strain L-14 Env during transfection

EBOV strain L-14 was titrated alongside 2000 ng of pSG3$^{\Delta env}$ during the transfection process. Produced virus stocks were tested for infectivity (technical repeats n= 3) in TZM-bl cells. Error bars represent mean with standard deviation.

To check if the low infectivity was a result of poor plasmid DNA, original plasmid DNA stocks of both EBOV strains were once more transformed and isolated from bacterial stocks. The newly produced plasmid stocks however, still yielded non-infectious virus.

An alternative transfection reagent, Lipofectamine 3000 was also used for transfection of these plasmids which resulted in non-infectious virus (data not shown). Following these experiments, the decision was made to proceed only with M-14, K-95, and MEM-14 plasmids.

3.2.2 General Assay characterisation

3.2.2.1 Comparison of Promega and Thermo Fisher luciferase activity detection kits

Both the Promega Luciferase assay system and the Thermo Fisher Luc-screen extended-Glow Luciferase Reporter Gene Assay System work on the principle, that Firefly luciferase a monomeric 61 kDa enzyme, catalyses the oxidation of the substrate luciferin using ATP. This generates light that is constant for approximately 1 min and can be detected by a luminometer. HEK293T cells were used to generate PV including K-95, MEM-14 and VSVg used as a positive control. The PV were infected onto TZM-bl cells and 48 h post infection luciferase activity was measured with the Promega and Thermo Fisher kit, results were

compared with the aim of identifying which kit had the greatest detection as well as sensitivity. Although no statistical significance was found possibly due to small sample size preventing statistical significance, a 10-fold greater detection was observed with the Promega luciferase assay system for VSVg infection as compared to the Thermo Fisher kit. The Promega kit was also deemed to be superior in the detection of K-95 and MEM-14 infected cells where the unpaired t-test showed statistical significance (P value= 0.0001 and 0.0022 respectively) over the Thermo Fisher assay system (Figure 3.11). The Promega kit had the greatest sensitivity for luciferase detection of the two kits tested and was therefore used in further experiments involving luciferase detection.

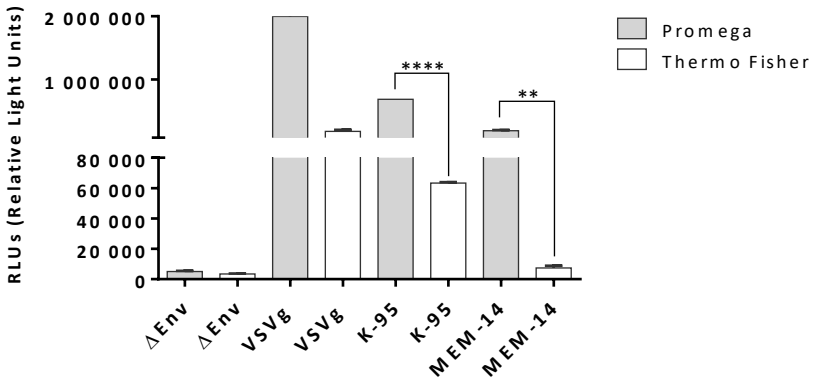


Figure 3.11 Comparison of two luciferase detection kits

EBOV virus stocks K-95, MEM-14 as well as VSVg were used for infection into TZM-bl cells. 48 h post-infection luciferase was detected using either the Promega (grey) or Thermo Fisher (white) luciferase detection kits. Significant differences (****P<0.0001 and **P<0.01) were observed in the infectivity of K-95 and MEM-14 (technical repeats n= 4) respectively with the Promega kit compared to the Thermo Fisher kit. Error bars represent mean with standard deviation.

3.2.2.2 Effect of plasmid stocks on viral infectivity

The efficiency of newly prepared plasmids to produce infectious virus stocks was evaluated. New pSG3$^{\Delta env}$ or EBOV GP plasmid stocks were validated by co-transfecting with an EBOV GP or pSG3$^{\Delta env}$ plasmid, respectively that had been utilised to produce all virus stocks thus far. Simultaneously, the well-utilised pSG3$^{\Delta env}$ and EBOV GP plasmids would be co-transfected together. The EBOV virus stocks produced from the parallel transfections would allow an accurate assessment of the proficiency of the new plasmid to produce infectious virus and allow a comparison of infectivity to the previous stock. If the results obtained from the

transfection and subsequent infection of TZM-bl cells demonstrated that the infectivity of virus produced with the new plasmid were comparable then these plasmids could be used to generate new virus stocks (see appendix).

3.2.2.3 Effect of variant PEI batches on transfection

PEI transfection reagent was freshly produced every three months. To ensure there were no problems with the PEI reagent, a transfection into HEK293T cells was carried out simultaneously with pSG3$^{\Delta env}$ and an EBOV plasmid using both the newly made and previously produced PEI. If the RLUs of the infected virus were in the same range (as illustrated in figure 3.8) the PEI was deemed validated for further use (see appendix).

3.2.2.4 Effect of HEK293T and TZM-bl cell passage number on transfection and EBOV infectivity

As immortalised cell lines are known to change morphology after a prolonged period in culture (PHE Technical support,. 2018), new cell lines were thawed from liquid nitrogen when cells in culture reached passage numbers in the late 40s. A newly thawed TZM-bl or HEK293T cell line was passaged several times prior to use. The cell lines with lower passage number were validated against the older cell line to ensure virus production and infectivity were comparable (see appendix).

3.2.3 Establishment and validation of an EBOV GP pseudo-typed HIV-1 neutralisation assay

Following the generation of the EBOV GP PV single-cycle system, we sought to assess the ability of utilising these stocks to measure EBOV plasma neutralisation.

3.2.3.1 Inhibitory range of EBOV pseudo-typed virus with EBOV negative human plasma

A comparative study assessed the neutralisation results of EBOV stocks by CP between different laboratories. It was observed that control plasma obtained from healthy donors also exhibited neutralising activity (Wilkinson et al., 2017). The negative sample from a UK donor had been tested and was unlikely to contain Abs to EBOV or to any constituent of the HIV-1 vector used by a participating laboratory. It was theorised that complement could play a role in neutralisation depending on the presence or absence of specific Abs, however, the non-specific inhibition of EBOV generally remained unexplained (Wilkinson et al., 2017). To

assess the extent to which healthy donor plasma could inhibit EBOV stocks, plasma samples donated from individuals unexposed to EBOV (n= 6) were serially diluted and incubated with EBOV stocks prior to infection into TZM-bl cells. At the most concentrated plasma dilution 1/32, K-95 and MEM-14 demonstrated up to 60% and 70% infectivity (Figure 3.12 A + B), respectively, thus from these inhibition profiles a negative control range was established (Figure 3.12 C).

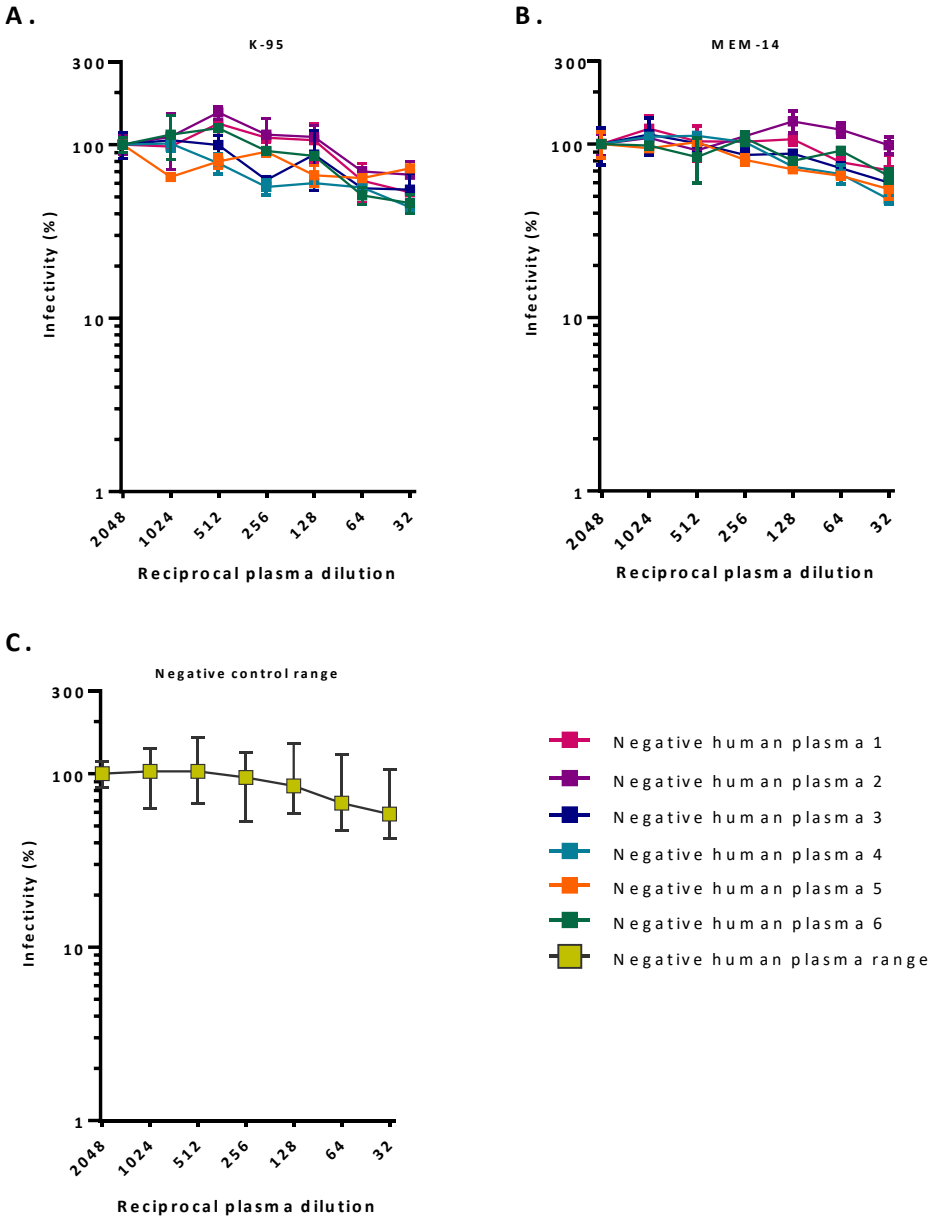


Figure 3.12 Incubation of EBOV virus strains with negative human plasma

A) K-95 and B) MEM-14 were incubated with Ebola negative human plasma (technical repeat n= 2) donated from 6 individuals prior to infection into TZM-bl cells. C) The neutralisation profiles of both strains were combined to give an acceptable negative control range with error bars representing mean with standard deviation.

3.2.3.2 Testing the specificity and performance of the EBOV-GP pseudo-typed assay

Following the production of the EBOV stocks, we sought to measure the ability of using these differing stocks to measure EBOV plasma neutralisation. The performance of the neutralisation assay was evaluated in comparison to a previous study carried out by the National Institute of Biological Standards and Control (NIBSC) (Wilkinson et al., 2017). The aim of their study was to establish a WHO International reference reagent for Abs against EBOV that could be used for the comparison and standardisation of serological assays, specifically PV neutralisation assays (Wilkinson et al., 2017). The range of International reference reagents available from NIBSC are detailed (Table 3.2).

Table 3.2 NIBSC Anti-EBOV convalescent plasma reference reagents

Information taken from (NIBSC., 2017 A, B and C)

WHO Plasma ID	Description	Reference
15/220	Anti-EBOV Zaire Convalescent human plasma. First WHO Reference Reagent- from an Ebola American Red cross survivor.	(NIBSC Medicines & Healthcare products, NIBSC Code: 15/220, 2017)
15/262	1st International Standard for EBOV antibodies Pooled convalescent plasma from six Sierra Leone patients recovered from EVD.	(NIBSC Medicines & Healthcare products, NIBSC Code: 15/262, 2017)
16/344	WHO Reference Panel, Anti-EBOV convalescent plasma Consisted of four EBOV convalescent plasma samples: 15/280, 15/282, 15/284 and 15/286 and one EBOV negative sample: 15/288.	(NIBSC Medicines & Healthcare products, NIBSC Code: 16/344, 2017)

The reference reagents were all analysed in the neutralisation assay with A) MEM-14, B) K-95 (Figure 3.13) and A) M-14 (Figure 3.14). Differential inhibition of the EBOV virus stocks was observed except with the negative human plasma; 15/288 from the WHO reference panel 16/344, which yielded results similar to the pre-established negative control range illustrated with the green box (Figure 3.14 A).

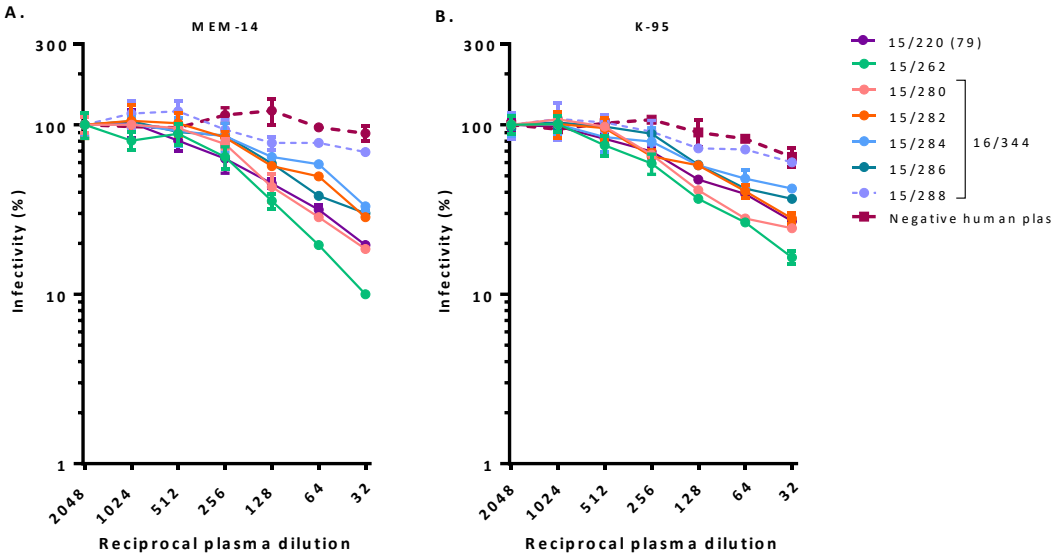


Figure 3.13 Incubation of EBOV strains with Anti-EBOV plasma from NIBSC

A) MEM-14 and B) K-95 EBOV strains were incubated with the range of NIBSC reference reagents; anti-EBOV human plasma, including reagent 15/220, 15/262 and the reference panel 16/344 prior to infection into TZM-bl cells (technical repeat n= 2). Luciferase activity was measured 48 h post-infection. Error bars represent mean with standard deviation.

In a previous study by NIBSC, filamentous EBOV like particles were incubated with the WHO International reference reagents 15/220, 15/284 and 15/282 (NIBSC 2017., A, B and C). For comparative purposes neutralisation IC_{50} values obtained from the NIBSC study were plotted alongside the IC_{50} values extracted from our neutralisation assay curve (Figure 3.14 A) with EBOV PV (Figure 3.14 B). The results show our neutralisation IC_{50} values are comparable to the results obtained by NIBSC and is therefore supportive of our methodology.

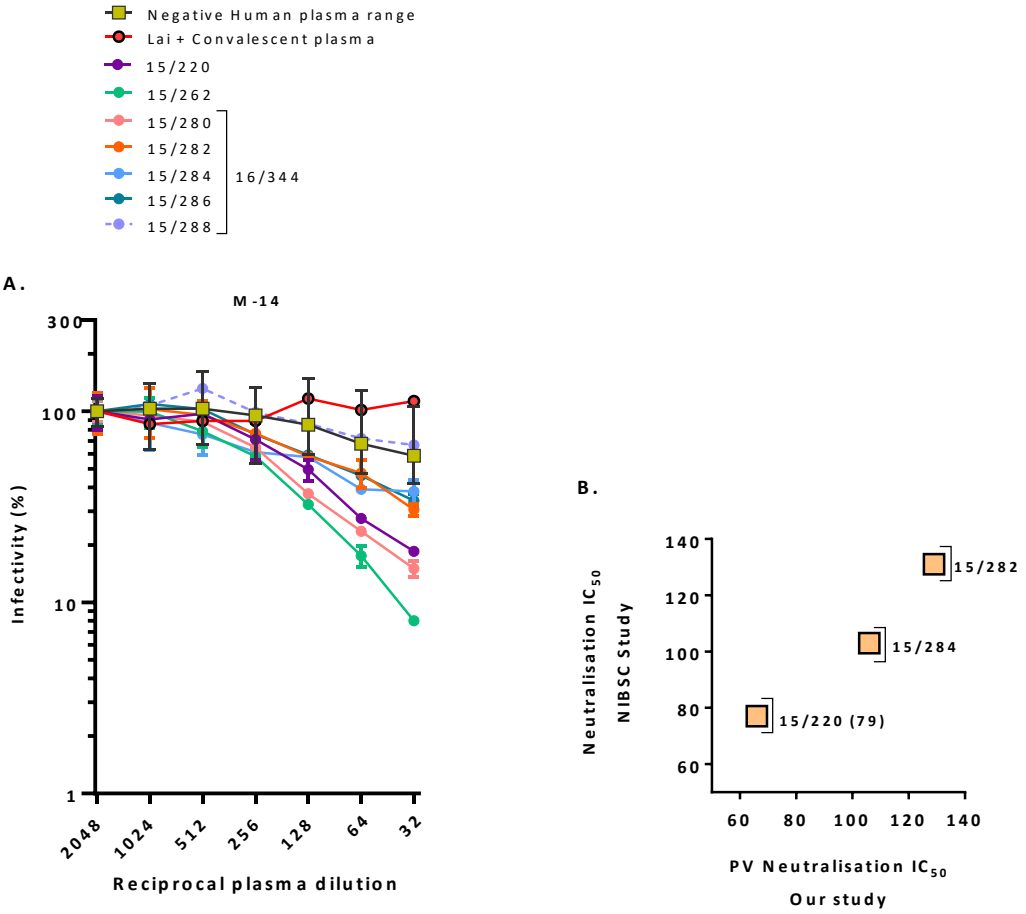


Figure 3.14 Neutralisation comparison between NIBSC and our study

A) EBOV strain M-14 was incubated with the NIBSC reference panel; anti-EBOV human plasma including reagent 15/220, 15/262 and 16/344 and CXCR4 HIV-1 pseudo-typed virus, LAI with EBOV survivor plasma (red circles) prior to infection into TZM-bl cells (technical repeat n= 2). Luciferase was determined 48 h post-infection with neutralisation profiles illustrated alongside the pre-determined negative human plasma range (green squares). Error bars represent mean with standard deviation. B) Neutralisation IC_{50} titres of EBOV strain M-14 incubated with the NIBSC reference reagents; 15/220, 15/284 and 15/282 compared to titres obtained from the Wilkinson *et al*, 2017 study.

The specificity of the assay was tested by incubating HIV-1 CXCR4 tropic PV with EBOV survivor plasma prior to infection of the TZM-bl cell line expressing both CCR5 and CXCR4 receptors. The infectivity of CXCR4 enveloped PV were not inhibited by EBOV survivor plasma indicating a lack of non-specific viral particle neutralisation shown as red circles with a black outline (Figure 3.14 A).

Likewise, the EBOV-GP PV was used to test the neutralisation potential of EBOV CP originating from a British nurse (referred to as W.P) who contracted Ebola during the 2013-2016 epidemic in Sierra Leone. W.P was treated with the experimental therapy ZMapp allowing him to make a speedy recovery. To examine how this treatment could affect the ability of his immune system to produce and retain Abs against EBOV GPs a neutralisation assay was conducted with plasma donated from W.P. As previously, described plasma was serially diluted and incubated with M-14 and K-95 prior to infection into TZM-bl cells and luciferase activity was measured 48 h post-infection. It was observed that the plasma was not efficient in preventing viral entry of both A) M-14 and B) K-95 (Figure 3.15). Most points of the neutralisation curves in both instances fall within the pre-determined negative control range for the assay. This suggests that W.P did not develop a robust adaptive immune response and was unable to retain Abs recognising the EBOV GP and therefore could not inhibit viral entry.

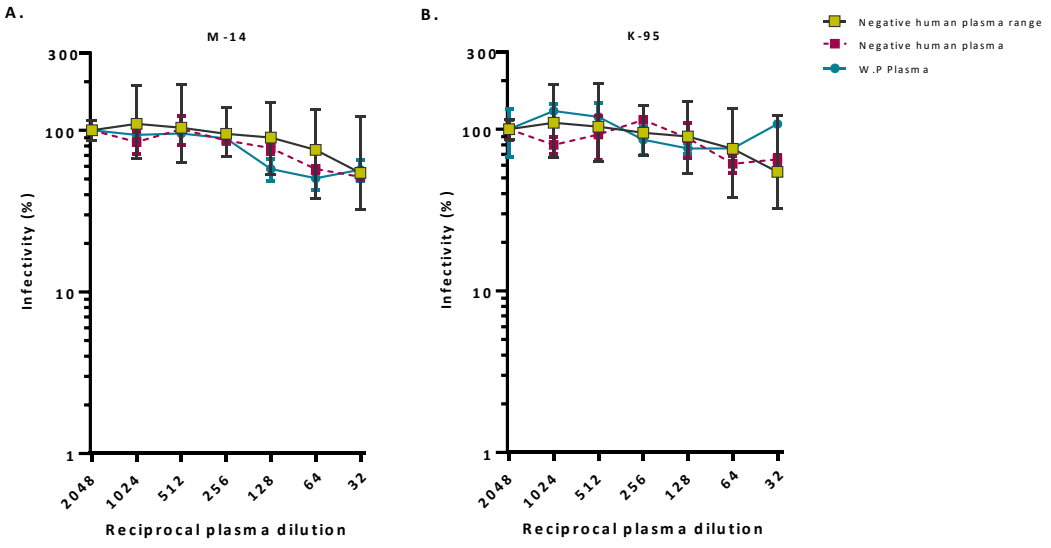


Figure 3.15 Neutralisaton of EBOV Strains with Ebola survivor CP

EBOV strains A) M-14 and B) K-95 incubation with negative human plasma (pink squares) and Ebola CP (teal circles) originating from a survivor treated with ZMapp prior to infection into TZM-bl cells. The predetermined negative human plasma range (green squares) is shown for comparative purposes. Error bars represent mean with standard deviation.

3.2.3.3 Testing the effect of different virus batches on neutralisation read out

As previously described an exclusion criterion was developed to ensure the infectivity of PV between batches remained within the determined range, also EBOV negative human plasma was run on each assay plate to control for variation between individual experiments. The effectiveness of these controls was tested during neutralisation repetition experiments between batches. Neutralisation experiments were carried out as previously described, EBOV PV was incubated with donor plasma prior to infection of TZM-bl cells and inhibition was measured by luciferase activity. Neutralisation experiments were repeated using the same donor plasma at different times and using variant batches of PV between the two experiments (Figure 3.16). Neutralisation titres obtained in the first experiment is shown as circles and the repeated corresponding experiment carried out with a different batch of PV is indicated by squares. The symbol colours identifies the different strain of EBOV PV used: yellow; M-14, purple; K-95 and green; MEM-14. Statistical analysis of the neutralisation IC_{50} data with non-parametric, Wilcoxon matched-pairs signed rank test showed no statistical difference between the data sets. These results further support the methodology and controls that have been put in place to promote standardisation of the neutralisation assay.

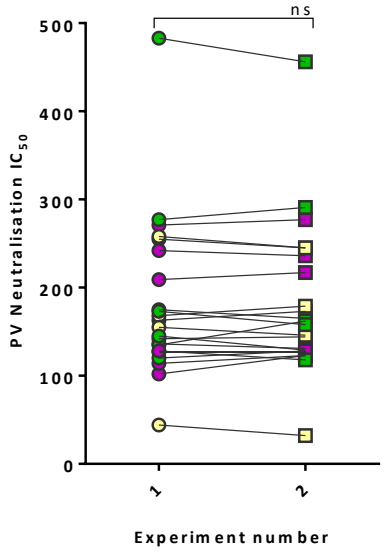


Figure 3.16 Different virus batch on neutralisation assay repetition

Neutralisation assays were carried out with EBOV CP and EBOV PV (yellow M-14, purple-K-95, and green-MEM-14). Neutralisation titres obtained in the first experiment with one batch of virus (circles) is shown with the corresponding repeated experiment carried out with a different batch of virus (squares). Significant differences were not observed with the non-parametric, Wilcoxon matched-pairs signed rank test.

3.3 Discussion

3.3.1 PV system selected

The HIV-1 retroviral packaging system was selected to produce the EBOV PV neutralisation assay, specifically the 2-plasmid system; one Env plasmid and one HIV-1 backbone plasmid. The retrovirus packaging system was chosen for the following reasons:

- Retroviral vectors can be pseudo-typed with wild-type EBOV efficiently (Sinn et al., 2017). The EBOV Env proteins are localised to the surface of the cell membrane, and HIV-1 packaging system would allow relatively easy generation and a high-yield of PV, as demonstrated previously in the literature for filovirus (Wilkinson et al., 2017).
- Both retroviruses and filoviruses assemble at the plasma membrane and have homotrimeric structural Env GPs mediating receptor binding and fusion. These similar properties may help produce efficient PV for phenotypic analysis (Sinn et al., 2017).
- The EBOV PV would ultimately be used to study virus entry and inhibition into host cell lines; the HIV-1 packaging system has been widely used for the production of EBOV stocks by many research groups for this purpose (J. Wang et al., 2014).
- Retroviral EBOV PV have been extensively used to study EBOV entry inhibitors (King and Tarr, 2017).

In addition, the luciferase reporter was chosen over the fluorescence reporter for use in the PV assay, reasons included:

- Higher sensitivity of readings (Liu et al., 2012).
- Ease of use.
- Shorter period required to process experiments with luciferase assay system than with FACs to detect fluorescence proteins.

A simplified, schematic drawing of A) EBOV PV production, B) infection and luciferase detection into TZM-bl cells and C) reduced luciferase detection in TZM-bl cells due to EBOV inhibition with NAbs in CP is depicted (Figure 3.17).

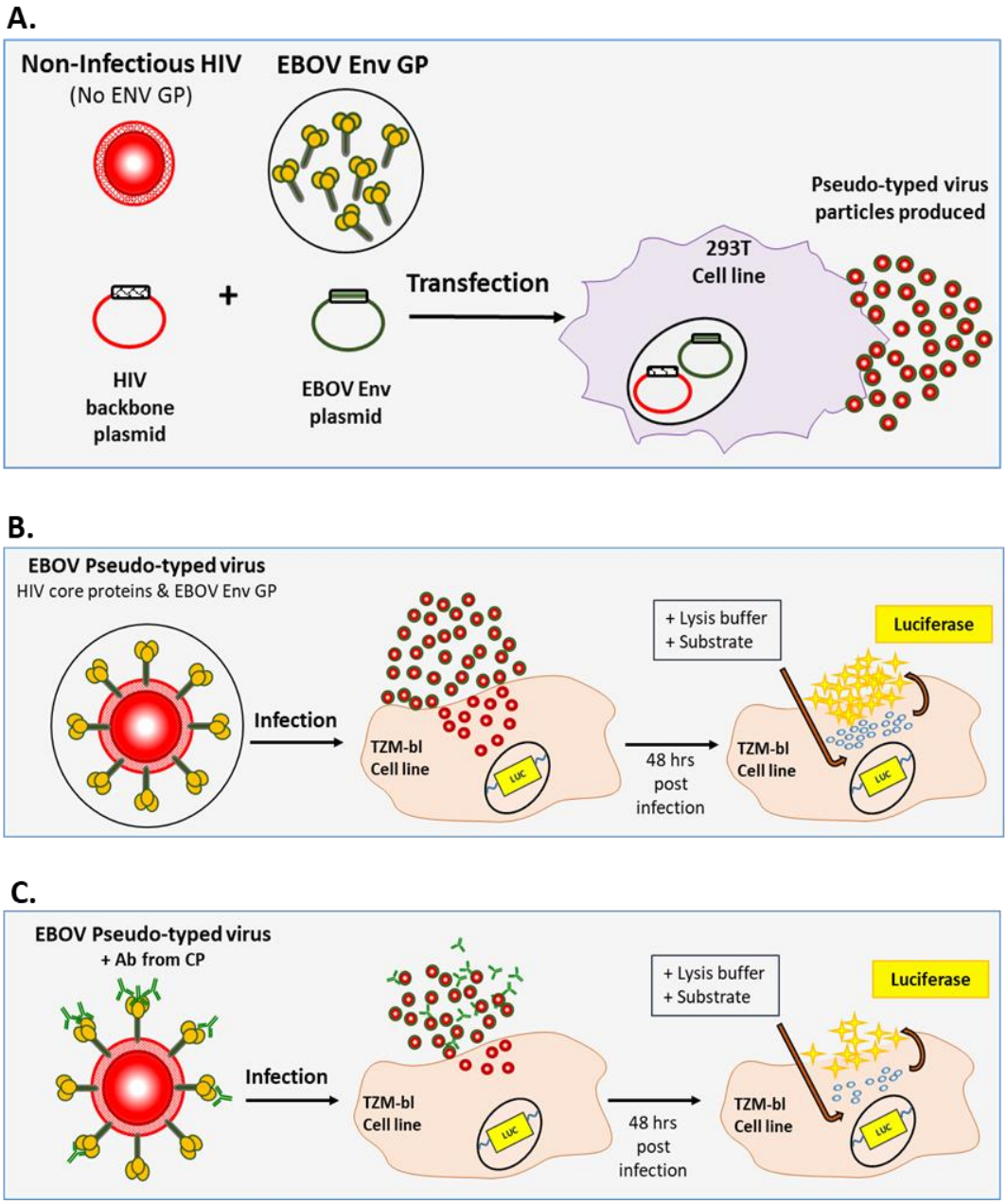


Figure 3.17 Schematic drawing showing production, luciferase detection and neutralisation of EBOV pseudo-typed virus in TZM-bl cells

A) The retroviral, HIV-1 vector deficient of the Env gene was pseudo-typed with EBOV Env GP. Both the HIV-1 backbone and EBOV GP encoded plasmids are transfected into HEK293T cell lines and EBOV PV were collected from culture supernatant. B) The produced EBOV PV were used for infection into the host cell line; TZM-bl and luciferase activity of the infected cells were examined post-infection. C) EBOV-GP specific Abs present in CP bind to the GPs, preventing entry of EBOV PV resulting in a reduction of luciferase activity.

3.3.2 Key optimisation experiments

Optimisation experiments were carried out in a logical process in order to deduce the best conditions for production of EBOV PV stocks for subsequent use in neutralisation assays with EBOV CP. Control experiments were conducted to evaluate the parameters of the assay for ease of use, assess limitations and to ensure reproducibility.

The range of EBOV Env input identified during transfection, which yielded similar virus infectivity, also revealed that p24 capsid production had very little variation within this range. Env input in EBOV PV production could be considered to be a steady variable; small differences in Env input would not greatly affect EBOV infectivity. Likewise it could be inferred that the number of EBOV virus particles produced would not differ substantially between virus batches made with the same amount of EBOV Env, therefore p24 was considered a constant during EBOV PV production.

A standard was developed to evaluate if newly produced EBOV PV fell within the accepted infectivity range for the respective virus strains. The exclusion criteria; the 95% percentile, calculated based on the infectivity of virus batches which did not show significant differences in their median, permitted the use of virus with fairly constant infectivity titres to be used in ensuing experiments. This operated as a control for both intra- and inter-infectivity variation between transfected dishes and batches of virus.

Many variations of EBOV PV and neutralisation protocols have been produced for the assessment of EBOV specific Ab neutralisation titres. The establishment of an international reference reagent for EBOV Abs by NIBSC allowed neutralisation data between laboratories to be more easily compared (Wilkinson et al., 2017). The similar neutralisation IC_{50} titres obtained between our study and the NIBSC study with the EBOV reference reagents supports the robust methodology developed here.

Controls such as similar PV infectivity titres used for neutralisation experiments as well as the EBOV negative human plasma range established as a negative control for each assay, permits the comparison of neutralisation data obtained between assay plates. The similarity between results of repeated neutralisation experiments, conducted with virus produced in different batches, further supports the methodology and controls that have been put in place

to promote a systematic analysis. The process of assay optimisation and validation provides assurance that assay results are as reliable as possible.

3.3.3 Chapter conclusions

The results presented in this chapter describe the successful development of a reliable neutralisation assay whilst assessing an extensive range of previously undocumented parameters that can potentially effect PV production, infectivity or neutralisation. This highlights the need for optimising protocols for each individual virus before assessing relative infectivity and performing inhibition assays (Urbanowicz et al., 2016a). The controls discussed in this chapter could be utilised in the systematic production of other types of PV. The development of this PV assay permits the evaluation of Ebola survivor CP NAbs from both a cross-sectional and longitudinal perspective as well as assessing the interaction of CP NAbs in the presence of EBOV soluble glycoprotein (sGP).

4 A cross sectional analysis of neutralising Abs of Ebola survivor convalescent plasma

4.1 Introduction

4.1.1 Immune responses

Survival of persons infected with EBOV requires the host to mount early and robust immune responses. The host's innate immune system is the first line of defence against Ebola which includes rapid detection of the foreign pathogen and activation of the host's innate immunity. Antigen-presenting cells (APCs); macrophages and dendritic cells (DCs) are significant players in the stimulation and regulation of innate immunity which studies have suggested; early, transient, controlled inflammatory response can help regulate replication and the spread of EBOV, leading to recovery from infection (Wong et al., 2014). Simultaneously these cells allow activation of the adaptive immune response which is predominantly responsible for elimination of the virus from the infected host as well as averting pathogen replication and spread. Abs are the product of the adaptive humoral immune response (Klasse, 2014). Rapid and vigorous humoral responses in survivors may help them to limit and control the dissemination of EBOV, consequently an impaired humoral response with no detectable specific IgG was associated with a fatal outcome for EBOV infected individuals (Baize et al., 1999).

4.1.2 NAbs

Much of the information of NAbs has been derived from research in to HIV-1 neutralisation. It has been documented that from a protective view, the most imperative antiviral role of Abs are neutralising pathogens, thereby preventing them from infecting cells (Payne, 2017). Neutralisation is mediated through the antigen recognition sites on the Ab structure known as the fragment antigen binding (Fab) region, targeting functionally active sites (Morris and Moody, 2016). There are many mechanistic aspects of neutralisation including how NAbs bind, the ability to induce conformational changes, whether they can irreversibly inactivate viral proteins mediating entry and whether their effectiveness is most prominent against virions in suspension or post virion-cell attachment (Klasse, 2014). However, here, neutralisation refers to the blocking of the early stages of the viral replicative cycle and is

defined as the capacity of an Ab to inhibit pathogen entry into a host cell. This can occur by preventing virions from attaching to target cells, blocking obligatory interactions necessary for virion entry, such as receptor engagement, endocytosis and fusion which may occur via mechanisms such as steric hindrance, target dissociation and the promotion of structural inflexibility in the pathogens surface proteins (Klasse, 2014; Morris and Moody, 2016).

It can take some time after an infection for a host to produce highly effective neutralising Abs, however, these may persist to protect the individual from future encounters with the virus (Payne, 2017). Therefore, as well as their roles in preventing, reducing and clearing infection, neutralising Abs can generally serve as a correlate of protection to viruses or other infectious pathogens (Morris and Moody, 2016).

4.1.3 EBOV NAbs in animal models and human studies

It has been demonstrated in NHPs and smaller animal models that adaptive immunity contributes to protection against EBOV and this immune protection seems to be linked with both induced humoral and cellular responses (Sobarzo et al., 2013). Some studies where this has been demonstrated include the protection of guinea pigs with a human anti-EBOV mAb, KZ52 (Maruyama et al., 1999b) (50 mg/ kg body weight), one hour after challenge with a high dose (10,000 PFU) of guinea pig-adapted EBOV. The high dose was chosen to represent high levels of viraemia ($10^{6.5}$ PFU/ ml of blood) that can be detected in human filovirus infection (Parren et al., 2002). Rhesus macaques were given a lethal, IM EBOV challenge (628 PFU) and were treated with ZMapp either 3, 6 and 9 dpi (Group 1), 4, 7 and 10 dpi (Group 2); or 5, 8 and 11 dpi (Group 3). All animals treated with ZMapp; a cocktail of three humanised mAbs, survived the infection compared to the control group who were given an IgG isotype control in place of ZMapp. Severe EBOV disease was reversed and high viraemia was controlled allowing for full recovery of the NHPs (Qiu et al., 2014). In another study IgG was purified from CP of NHPs that were challenged with EBOV (1000 PFU). Subsequently, naïve NHPs were EBOV challenged (1000 PFU) and administered the species-matched polyclonal IgG 48 h post-infection, the purified IgG was found to confer protection to other NHPs against ZEBOV post-exposure (Dye et al., 2012).

The benefit of NAbs in relation to humans was assessed during the 2013-2016 Ebola outbreak. The World Health Organisation (WHO) supported the use of experimental EVD treatments which permitted the fast-tracking of non-randomised clinical trials using whole

blood or plasma infusion to treat patients with EVD (Harris, 2015). The most comprehensive trial; the Ebola-Tx trial (ClinicalTrials.gov number NCT02342171), transfused 84 EBOV infected patients with two consecutive transfusions of 200-250 ml ABO compatible CP with unknown Ab titres, each provided by a separate donor (van Griensven et al., 2016b). Post-trial analysis, indicated that although associations were not significant, mortality was lower in the CP group compared to the control group, higher IgG doses resulted in lower mortality, whilst higher levels of NAbs resulted in higher mortality (van Griensven et al., 2016a). Conclusions of the trial included; unknown levels of NAbs present in CP at time of administration was a limiting factor and if NAbs were crucial in CP therapeutics, an Ebola-GP PV assay should be developed for on-site use (Cardile et al., 2017).

To date there have been limited studies on naturally occurring EBOV NAbs produced in humans. Studies thus far have included research utilising KZ52, the mAb isolated from a survivor of the 1995 Kikwit outbreak and more recently Abs in whole blood or CP have been assessed in clinical trials as a potential therapeutic for EVD. To our knowledge this is the first study of its kind to explore EBOV specific NAb titres present in EBOV CP in a large cohort.

4.1.4 Aims of this chapter

The results in this chapter aim to explore associations between the neutralisation capacities of anti-EBOV Abs present in CP of Ebola survivors with various donor related parameters by undertaking a cross-sectional analysis. The principal characteristic of the cross-sectional analysis is that all measurements taken from a sample member are acquired at a particular time point over a designated recruitment period. Cross-sectional studies can provide information on the prevalence of disease and examine trends between health related characteristics and other variables of interest (Sedgwick, 2014). EBOV NAb titres were analysed in relation to the following parameters:

1 Survivors within the same cohort
2 Gender of the donors
3 Age of the donors
4 Body Mass Index (BMI) of the donors
5 Disease severity of the donors
6 Time post convalescence the CP sample was donated

4.2 Results

4.2.1 Ebola survivor cohort

4.2.1.1 Recruitment of cohort

Plasma samples donated from Ebola survivors of the 2013-2016 outbreaks were recruited from Freetown, Sierra Leone as part of the study "Convalescent plasma for early Ebola virus disease in Sierra Leone"; subsequently CP samples of the donors were used here. The recruitment process for volunteers included EVD survivors with certificates (issued on discharge by the Ebola treatment centre) first being seen at the 34th Regimental Military Hospital, Wilberforce Freetown (MH34). 130 survivors were recruited on average 6.1 months (range 1-15, mid quartiles 4-8) post discharge, 12 were referred to the Ebola Survivors' Clinic due to possible post EVD sequelae (Scott et al., 2016). 118 healthy volunteers were referred to the Blood Bank, National Safe Blood Service, Connaught Hospital Freetown, however, three donors were excluded due to hepatitis B virus infection. The remaining 115 became consenting, qualified donors, providing 315 plasma samples (Richard S. Tedder et al., 2018). The recruitment process is summarised in Figure 4.1.

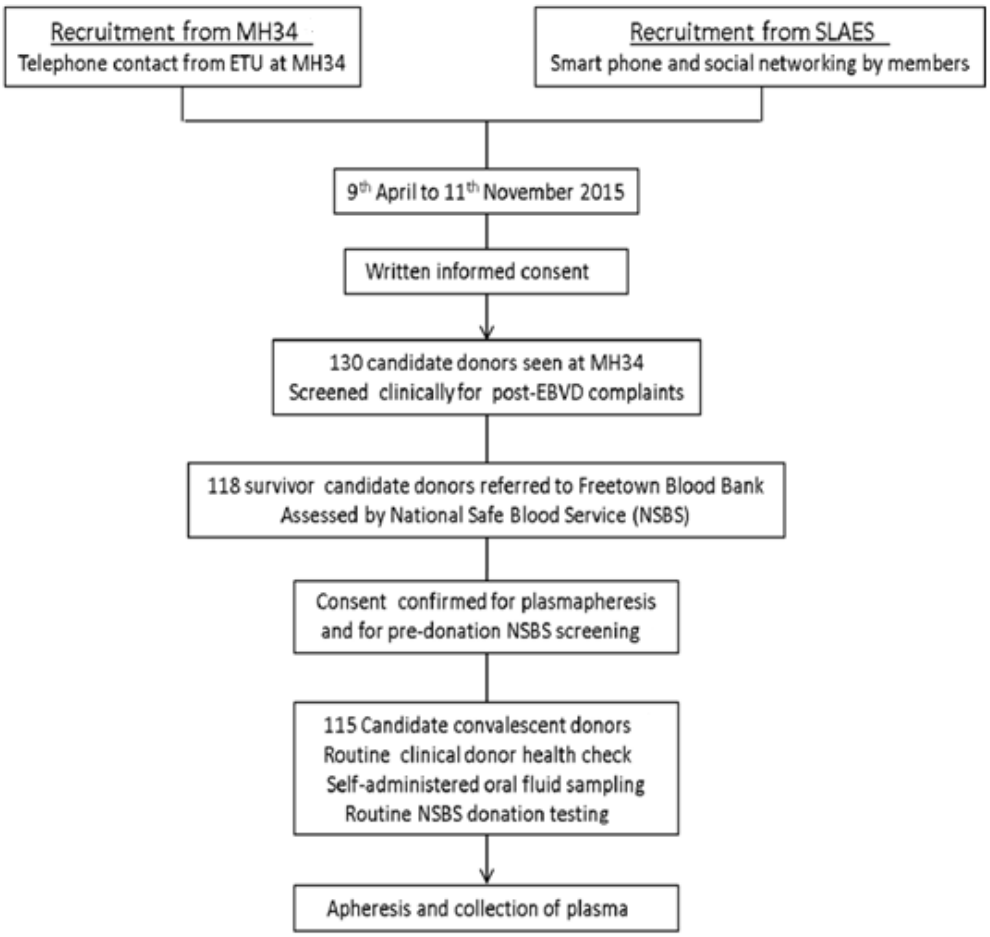


Figure 4.1 Ebola survivor recruitment

Summary of the recruitment process for Ebola survivors as part of the study "Convalescent plasma for early Ebola virus disease in Sierra Leone". Taken from (R.S. Tedder et al., 2018)

4.2.1.2 Sub-population of cohort

From the cohort recruited as part of the study "Convalescent plasma for early Ebola virus disease in Sierra Leone", a sub-population of donors were selected for measuring the titres of EBOV NAb in CP using PV trans-complemented with the EBOV GP.

The selection process was based on:

1. The time elapsed between the discharge date of the donor from the clinic which is also referred to as Day 0 of convalescence (the day the survivor tested negative for Ebola for a second time) and the first plasma sampled.
2. Donors who provided multiple samples over a period of time.

3. Donors who provided samples closer to their discharge date were prioritised however samples were also selected across the range till the last sample available was donated, in order to analyse how time may affect EBOV NAbs.

Donors that were selected for our study population are listed in Table 4.1 including their corresponding clinical parameters such as gender, age, BMI and severity of disease. BMI was recorded during the study recruitment process and disease severity was stated by the donor comparatively to others who had suffered from EVD. The time the CP sample was donated post convalescence is also listed in Table 4.1. Further details of the cohort are summarised (Table 4.2) including the CP samples distributed by gender, age, BMI, disease severity and sampling time.

For the cross sectional study, the earliest CP sample donated from a donor with corresponding neutralisation IC_{50} titres for all three EBOV strains; M-14, K-95 and MEM-14, were chosen for analysis (N= 46).

Table 4.1 Donor demographic parameters of cohort

BMI was assessed during the study recruitment process and severity of disease was stated by the donor in comparison to others who suffered EVD, 3= severe, 2= moderate, 1= mild and n= no data. EVD severity and days post convalescence when the plasma sample was donated. Convalescence was standardised; day 0= donor tested double negative for EBOV.

Donor ID	Sex	Age	BMI	Comparative Severity	Days post convalescence
IGH_EBOV CP Donor_001	Male	32	24.7	n	333
IGH_EBOV CP Donor_002	Male	52	21.6	n	248
IGH_EBOV CP Donor_003	Male	36	21.5	n	241
IGH_EBOV CP Donor_004	Male	30	19.3	n	263
IGH_EBOV CP Donor_005	Male	30	22.3	n	256
IGH_EBOV CP Donor_006	Female	29	27.7	n	247
IGH_EBOV CP Donor_007	Male	22	24.6	n	251
IGH_EBOV CP Donor_008	Male	27	21.8	n	228
IGH_EBOV CP Donor_009	Male	43	24.8	n	141
IGH_EBOV CP Donor_010	Male	27	24.1	n	138
IGH_EBOV CP Donor_011	Male	27	20.3	n	257
IGH_EBOV CP Donor_012	Male	27	26.1	n	290
IGH_EBOV CP Donor_013	Female	34	26.6	n	216
IGH_EBOV CP Donor_014	Male	37	22.7	n	169
IGH_EBOV CP Donor_015	Male	26	21.9	n	229
IGH_EBOV CP Donor_016	Male	22	24.5	n	208
IGH_EBOV CP Donor_017	Male	25	24.6	n	252
IGH_EBOV CP Donor_018	Female	42	25.1	n	262
IGH_EBOV CP Donor_019	Female	40	22.2	n	226
IGH_EBOV CP Donor_020	Female	38	20.2	n	305
IGH_EBOV CP Donor_021	Male	21	22.3	n	274
IGH_EBOV CP Donor_022	Male	21	20.9	n	325
IGH_EBOV CP Donor_023	Male	25	23.8	n	115
IGH_EBOV CP Donor_024	Female	24	25.3	n	112
IGH_EBOV CP Donor_025	Male	19	20.2	n	200
IGH_EBOV CP Donor_026	Female	26	25.2	n	145

Donor ID	Sex	Age	BMI	Comparative Severity	Days post convalescence
IGH_EBOV CP Donor_027	Female	25	28.3	n	173
IGH_EBOV CP Donor_028	Male	35	29.1	n	156
IGH_EBOV CP Donor_029	Male	40	21.7	1	33
IGH_EBOV CP Donor_030	Male	35	19.1	n	31
IGH_EBOV CP Donor_031	Female	25	23.5	3	34
IGH_EBOV CP Donor_032	Female	22	23.2	2	31
IGH_EBOV CP Donor_033	Female	22	32	2	31
IGH_EBOV CP Donor_034	Female	22	24.3	1	99
IGH_EBOV CP Donor_035	Female	21	21.5	2	217
IGH_EBOV CP Donor_036	Male	28	23.2	1	48
IGH_EBOV CP Donor_037	Male	18	23.1	3	236
IGH_EBOV CP Donor_038	Male	33	20.8	1	61
IGH_EBOV CP Donor_039	Female	21	20.8	3	91
IGH_EBOV CP Donor_040	Male	28	21.8	1	164
IGH_EBOV CP Donor_041	Male	20	22.9	2	175
IGH_EBOV CP Donor_042	Female	27	25.4	2	244
IGH_EBOV CP Donor_043	Male	38	21.5	1	179
IGH_EBOV CP Donor_044	Male	19	26.7	3	91
IGH_EBOV CP Donor_045	Female	18	21.3	3	186
IGH_EBOV CP Donor_046	Male	42	23.1	2	220
IGH_EBOV CP Donor_047	Male	23	20.1	2	192
IGH_EBOV CP Donor_048	Male	23	24.2	1	196
IGH_EBOV CP Donor_049	Male	22	20.7	2	261
IGH_EBOV CP Donor_050	Male	18	20.5	2	179
IGH_EBOV CP Donor_051	Male	22	22.6	1	192
IGH_EBOV CP Donor_052	Female	33	30.8	2	277

Table 4.2 Distribution of donor demographic parameters of the cohort

Cohort details in relation to clinical parameters as well as CP sampling time post convalescence.

Cohort details	Intervals	Total (n)
Cohort donors		52
Total CP samples used in cross-sectional study		52
Sex	Male	35
	Female	17
Age (years)	18-21	10
	22-25	14
	26-30	12
	31-35	6
	36-40	6
	41-45	3
	46-50	
	51-55	1
	56-60	
	> 61	
Body Mass Index (BMI)	< 18.5	0
	18.5 - 24.9	42
	25 - 29.9	8
	30 - 39.9	2
Disease severity	No data	29
	Mild	8
	Moderate	10
	Severe	5
Sampling number post convalescence (days)	< 100	10
	100-199	16
	200-299	23
	300-399	3
	≥ 400	0

4.2.2 Neutralisation Curves

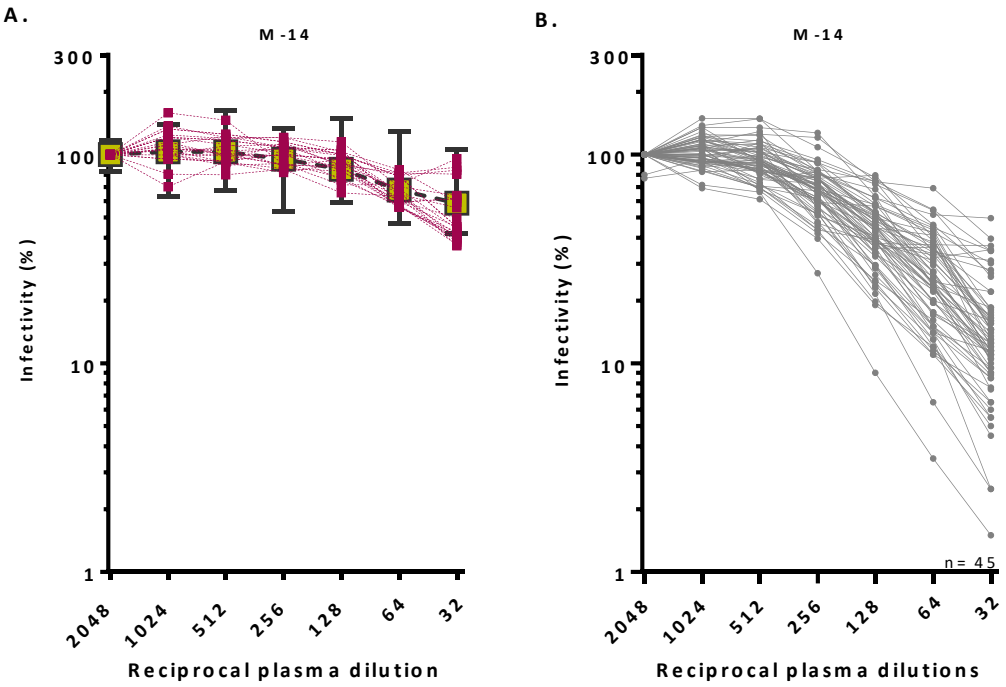
Conventionally measurements of potency and breadth of NAbs that inhibit a specific virus input, are determined by the Ab concentration that inhibits either 50% (IC_{50}) or 80% (IC_{80}) of the virus in a dose-response single-cycle infection assay *in vitro* (Webb et al., 2015). Due to the wide-ranging EBOV neutralisation by CP that was observed, values were extracted from the neutralisation curves at Ab inhibition IC_{50} for further analysis in order to incorporate maximum data points.

4.2.2.1 Wide range of neutralisation potential of EBOV Abs

Following the validation of our EBOV PV neutralisation assay against the NIBSC study (Figure 3.14 B) which, established a WHO International reference reagent for Abs against EBOV, we sought to assess the neutralisation potential of CP donated from our Ebola survivor cohort. As previously described, the neutralisation assay was carried out by first serially diluting the EBOV negative human plasma and CP samples; ranging from 1/32 to 1/2048 dilution. CP from donors were incubated with the different EBOV virus strains; M-14 (n= 50), K-95 (n= 49) and MEM-14 (n= 48) which was used for infection of TZM-bl cells. The neutralisation of EBOV negative human plasma run parallel on each assay plate was used as a validation control for the experiment.

Neutralisation was not observed for any of our plasma samples at dilutions 2048 and 1024 and were deemed non-neutralising dilutions. For uniformity, results were expressed as a reciprocal of the highest dilution (2048) (Truelove et al., 2016), which was considered the best condition to use as a control to assess neutralisation for each plasma dilution/sample, as this provided for a lower degree of variation. For comparative purposes luciferase activity of cells infected with virus alone was used to compare corresponding virus pre-incubated with a subset of CP samples (n= 30 donors). The difference in RLUs were expressed as a percentage of infection and were presented as neutralisation curves, where the results revealed no significant differences in neutralisation IC_{50} titres acquired when using either method (see appendix). EBOV negative human plasma incubated with A) M-14, C) K-95 and E) MEM-14 shows virus inhibition (pink squares/dotted lines) within the accepted pre-determined assay control range (green squares) (Figure 4.2). This allowed for acquired data of EBOV neutralisation with CP to be used for further analysis.

The neutralisation curves obtained from donor CP incubated with B) M-14, D) K-95 and F) MEM-14 was incorporated into one graph, in order to compare the neutralisation potential of anti-EBOV Abs present in the CP samples (Figure 4.2). Neutralisation curves for individual CP samples are the average of technical repeats (n= 2) and biological repeats (ranging from n= 1 to n= 5). Comparison of the neutralisation curves revealed a wide range of CP neutralisation potential which was observed for each of the EBOV strain.



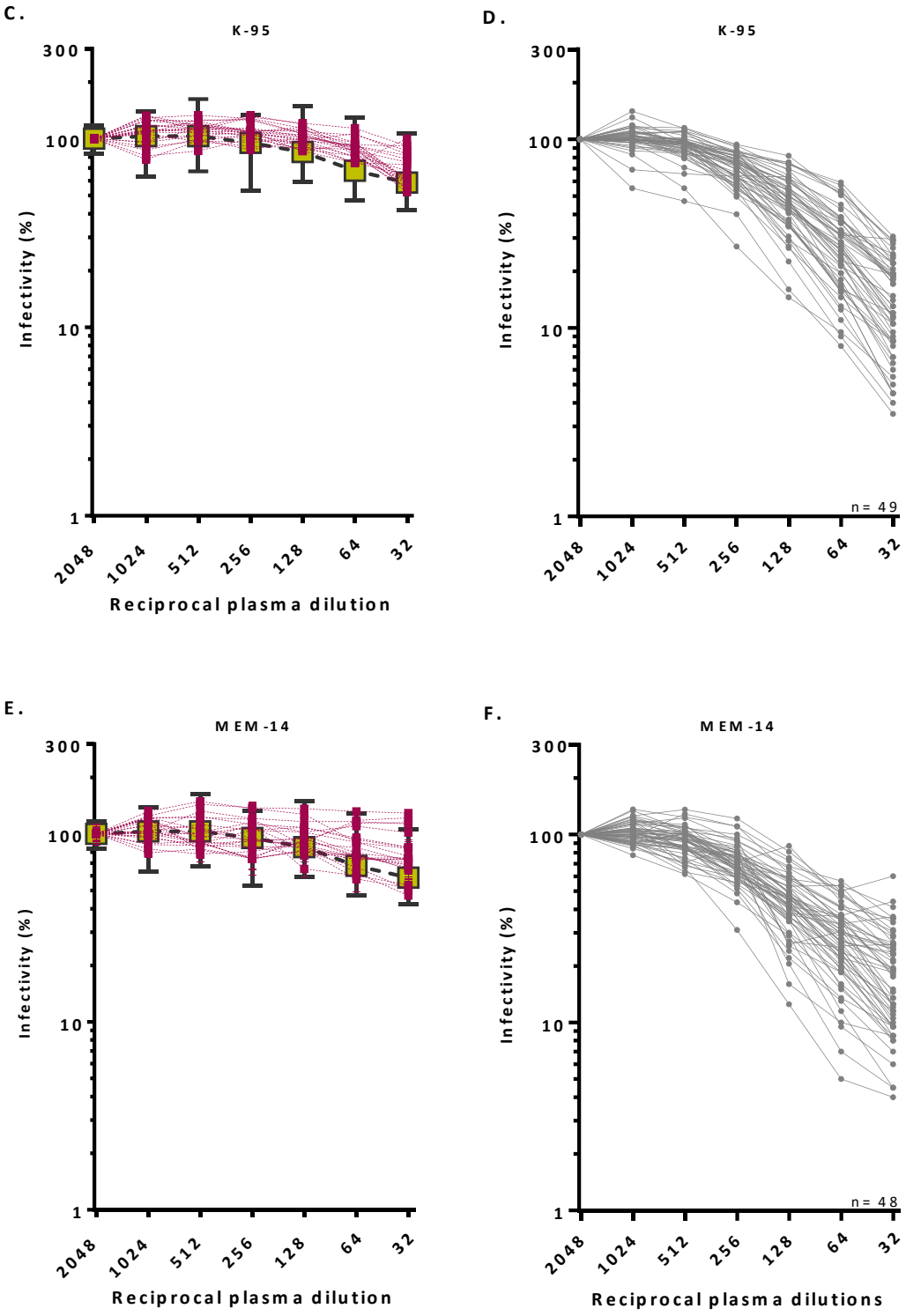


Figure 4.2 Wide neutralisation range of anti-EBOV Abs

Infectivity of the negative control; EBOV negative human plasma (pink squares/ dotted lines) incubated A) M-14, C) K-95 E) MEM-14. Green square denotes pre-determined negative human plasma range for assay. Infectivity of EBOV PV strains B) M-14 (n= 45), D) K-95 (n= 49) and F) MEM-14 (n= 48) incubated with CP prior to infection into TZM-bl cells (grey lines). Results shown as a reciprocal of the highest Ab dilution and as a percentage of infection (Y axis). Error bars indicate mean with standard deviation.

4.2.3 Comparison of EBOV NAb titres between EBOV strains

Two different strains of EBOV, M-14 and K-95 were utilised in the aforementioned study to assess the capacity of 2014 CP to neutralise virus strains which came into circulation nearly 20 years apart. MEM-14 a variant of M-14 was utilised to assess the capacity of CP to neutralise a homologous EBOV strain which could have potentially undergone GP mutations. Neutralisation IC_{50} values extracted from neutralisation curves of K-95, M-14 and MEM-14 incubated with 2014 CP were plotted on dot blots to evaluate neutralisation differences between the two virus strains and the variant. The analysis revealed that between EBOV PV strains there were CP samples that had similar neutralisation IC_{50} titres and CP samples in which the titres varied (see appendix), however, an overall comparison of the neutralisation IC_{50} titres revealed no significant differences between the three strains compared (Kruskal-Wallis test) (Figure 4.3 A). This indicates the CP which was homologous to the 2013 EBOV strain had a similar capacity to neutralise the three variant EBOV strains, this was further iterated when a non-parametric Spearmann correlation analysis was applied between the neutralisation IC_{50} data sets of M-14 and K-95 (Figure 4.3 B) and MEM-14 (Figure 4.3 C) respectively. A statistically significant correlation ($P<0.0001$) in both comparisons was maintained indicating that the 2014 CP samples were as potent at neutralising the different EBOV strains; K-95 and MEM-14 as it was at neutralising the homologous EBOV strain M-14.

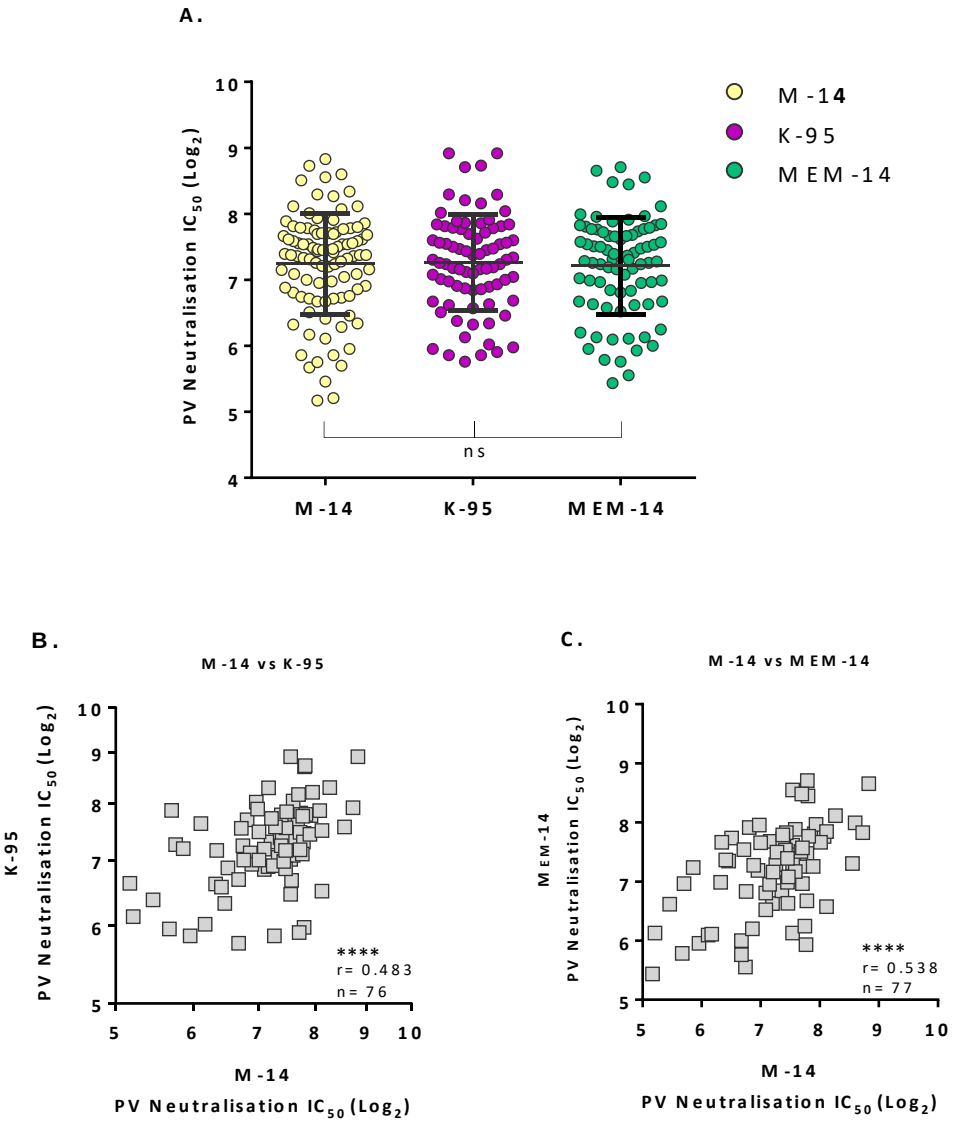


Figure 4.3 Comparison of CP NAbs titre of K-95, M-14 and MEM-14

A) Neutralisation IC_{50} titres obtained from M-14, K-95 and MEM-14 with CP were analysed by Kruskal-Wallis test yielding no significant differences. Neutralisation IC_{50} titres of B) K-95 and C) MEM-14 were plotted against M-14 respectively. A non-parametric Spearmann correlation revealed statistically significant correlation (****P<0.0001) between both comparisons. Error bars indicate mean with standard deviation.

4.2.4 EBOV NAb analysis according to donor demographics

The inhibition profiles of the different EBOV stocks with CP illustrated that donors from the cohort had differing neutralisation potential. To evaluate if these differences were the result of individual donor characteristics, IC_{50} values extracted from the neutralisation curves were converted to log_2 and were categorised according to virus strains and specific donor related parameters to observe if any trends could be detected.

4.2.4.1 Neutralisation potential of Anti-EBOV Ab distinguished by gender of cohort

Physiological differences between males and females has been demonstrated with differential susceptibility and responses to a number of different infectious diseases (Klein, 2012), including viral, bacterial and parasitic infections (Van Lunzen and Altfeld, 2014). The intensity, prevalence and pathogenesis of viral infections has been noted to be different between the sexes (Klein, 2012). These variances are attributed to many reasons including different exposure to common pathogens, hormonal factors; altering susceptibility or disease progression and immune responses against pathogens modulated by genetic factors and treatment responsiveness (Van Lunzen and Altfeld, 2014). Progesterone is known to activate the progesterone response element (PRE) of the human papillomavirus (HPV). Activation of the PRE regulates part of the HPV life cycle and transformation process which may explain the higher frequency of malignant HPV lesions in females compared to males (Chan et al., 1989) thus illustrating how hormonal factors can affect disease susceptibility. An example of genetic factors affecting immune responsiveness are the genes that are central to the regulation of immunity including Toll-like receptors 7 and 8 that play an important role in sensing viral pathogens, FOXP3; a transcription factor for regulatory T cells which are encoded by the human X chromosome (Van Lunzen and Altfeld, 2014). However, currently there is no data relating to how the gender of an individual can relate to the neutralisation capacity of the CP anti-EBOV Abs.

To evaluate if there was a difference in EBOV neutralisation by CP attributed to gender within the cohort, IC_{50} values were presented as a dot blot, and grouped by virus strain as well as gender of the donors, males (n= 35) and females (n= 17) (Figure 4.4 A). A non- parametric, Mann-Whitney U test indicated there were no significant differences between EBOV Ab titres in males and females within the same virus strain.

To assess potential differences between EBOV Ab neutralisation of M-14, K-95 and, MEM-14, IC_{50} titres were analysed within the female and male participants with a non- parametric, Kruskal-Wallis test which also revealed no significant differences between the virus strains (Figure 4.4 A). The compiled neutralisation titres of the three EBOV strains grouped into two data sets; females and males were analysed with an unpaired, non-parametric t-test indicating there were no significant differences overall in IC_{50} titres between the genders (Figure 4.4 B). These results suggest that gender may not be an independent factor affecting the titres of EBOV NAbs present in CP.

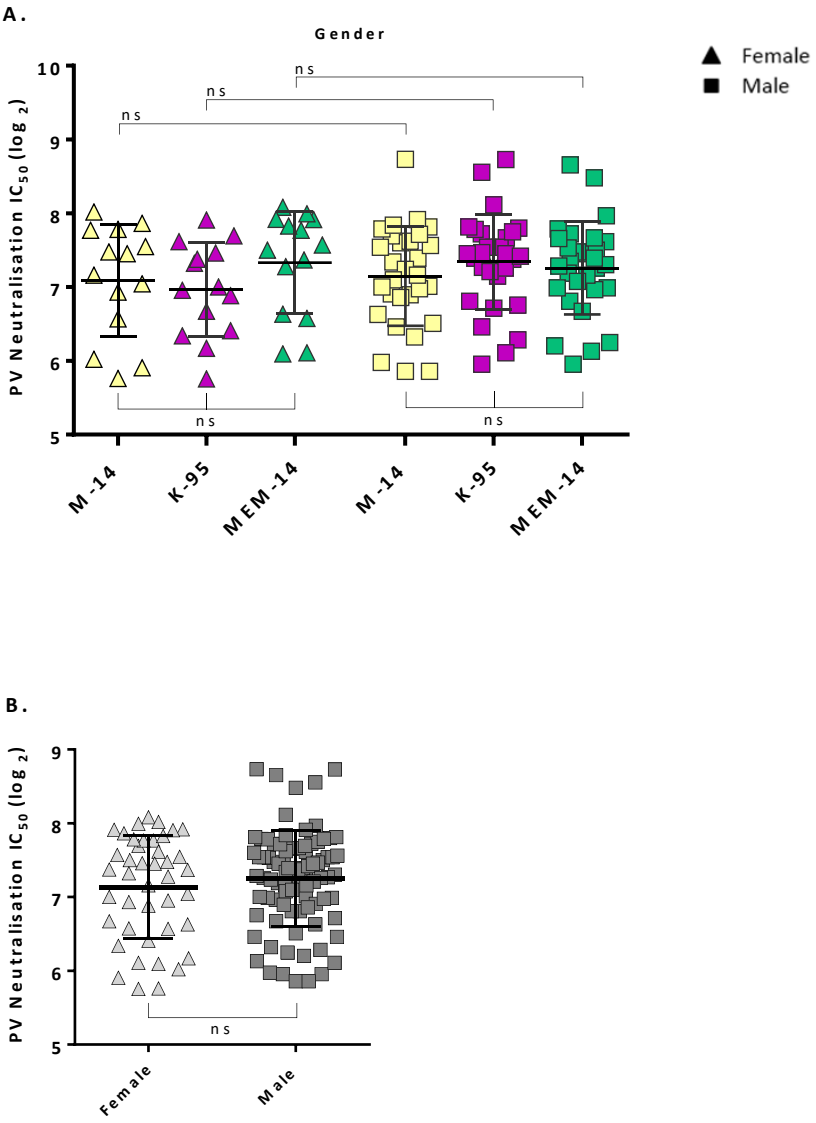


Figure 4.4 Neutralisation IC_{50} of EBOV strains categorised by Sex of cohort

A) A non-parametric Mann-Whitney U test was used to analyse differences between males and females of each EBOV strain. The difference in neutralisation titres for M-14 (yellow), K-95 (purple) and MEM-14 (green) EBOV strains were compared to each other in both the male and female cohort with a non-parametric Kruskal-Wallis test. B) Neutralisation titres of the three EBOV strains were compiled for both females and males and were assessed for differences with a Mann-Whitney U test. No statistical significance was observed for either tests. Error bars represent mean with standard deviation.

4.2.4.2 Comparison of two age groups on EBOV Ab neutralisation capacity

Aging, profoundly changes the immune and endocrine systems which can increase susceptibility to infectious diseases (Gavazzi and Krause, 2002). Immunosenescence affects both the innate and adaptive immune systems, the capacity for immune cells to function declines with age (Giefing-Kröll et al., 2015). In both men and women of advancing age it is apparent that the abilities to mount sufficient Ab responses reduces, specially towards new antigens (Giefing-Kröll et al., 2015). Likewise young infants and children are also at a high risk of pathogenic viruses, bacteria, fungi and parasites due to their relatively immature immune systems (Simon et al., 2015). Vaccine studies can provide insights as to how age may affect NAb titres. In a study that looked at the functional characteristics of Ab responses induced by an inactivated flavivirus vaccine against tick-borne encephalitis (TBE) in young and elderly healthy volunteers, they found no differences in the avidity and functional activity of induced Abs, however, the elderly were found to produce lower titres (Stiasny et al., 2012). A study analysing the effect of age on NAb responses in HIV-1 gp120 vaccine recipients found no measureable effect on NAb responses (Montefiori et al., 2004). Another study assessing the effect of age on influenza vaccine responses found that being older had a strong effect on induced vaccine response; with low seroprotection rate as well as decreased neutralising functionality of vaccine induced Abs being reported (Olafsdottir et al., 2018).

In order to evaluate if there were any noticeable differences between a lower (n= 26) and higher (n= 18) age range and EBOV neutralisation titres, IC_{50} values were grouped by EBOV strain as well as into two age groups. The age groups were separated based on the median age of the cohort, 27 years. The data, represented as a dot blot did not pass a normality and variance test therefore a non-parametric Mann-Whitney U test was used to compare any differences in IC_{50} values between the two age groups within the same EBOV strain. No significant differences were observed between IC_{50} titres of the two groups for M-14 and MEM-14, however, a significant difference (P= 0.0036) was seen for K-95. The difference in neutralisation titres of the three EBOV strains were compared to each other within each age group by the Kruskal-Wallis test, though no significant differences were observed, indicating virus strain did not impact NAb titres within the same age group (Figure 4.5).

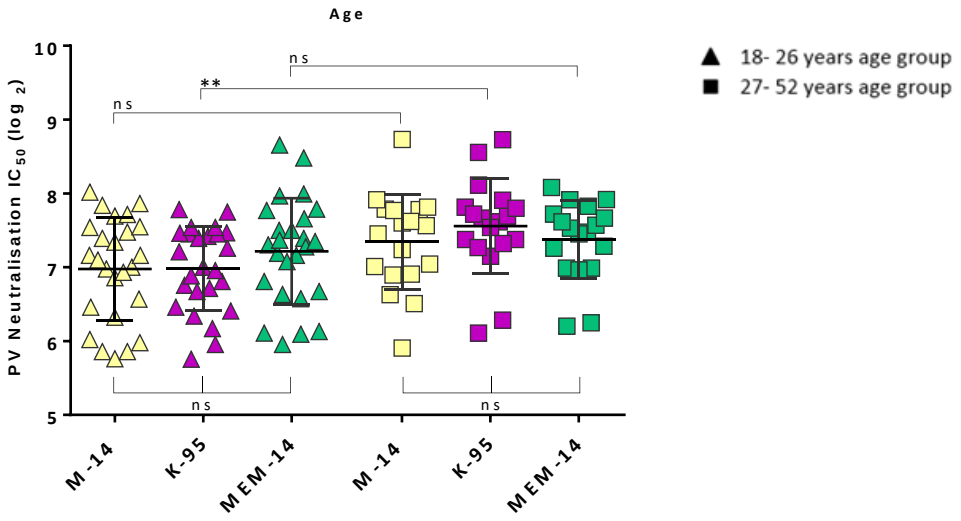


Figure 4.5 Neutralisation IC_{50} of EBOV strains grouped into two age groups

Age groups were separated based on the median value and neutralisation IC_{50} titres were compared between the two age groups for the same virus strain with a non-parametric Mann-Whitney U test. No statistical significance was observed for M-14 and MEM-14, K-95 showed a statistical difference (**P<0.01) between the groups. A Kruskal-Wallis test was used to analyse differences between the EBOV strains M-14, K-95 and MEM-14 within each age group, no statistical differences were observed. Error bars represent mean with standard deviation.

4.2.4.2.1 Effect of age on EBOV NAb titres

The neutralisation values for all donors within the cohort were plotted against their respective age for each EBOV strain; M-14, K-95 and MEM-14 in order to evaluate if there were any overall trends between age and the viral inhibitory capacity of EBOV NAbs. Statistical analysis of the graphs with linear regression analysis showed that EBOV NAb titre was not dependent on age for C) MEM-14, however the r^2 for A) M-14 and B) K-95 were 0.1329 and 0.1219 respectively (Figure 4.6). This indicates age is a very weak predictor of NAb IC_{50} titres for these two strains.

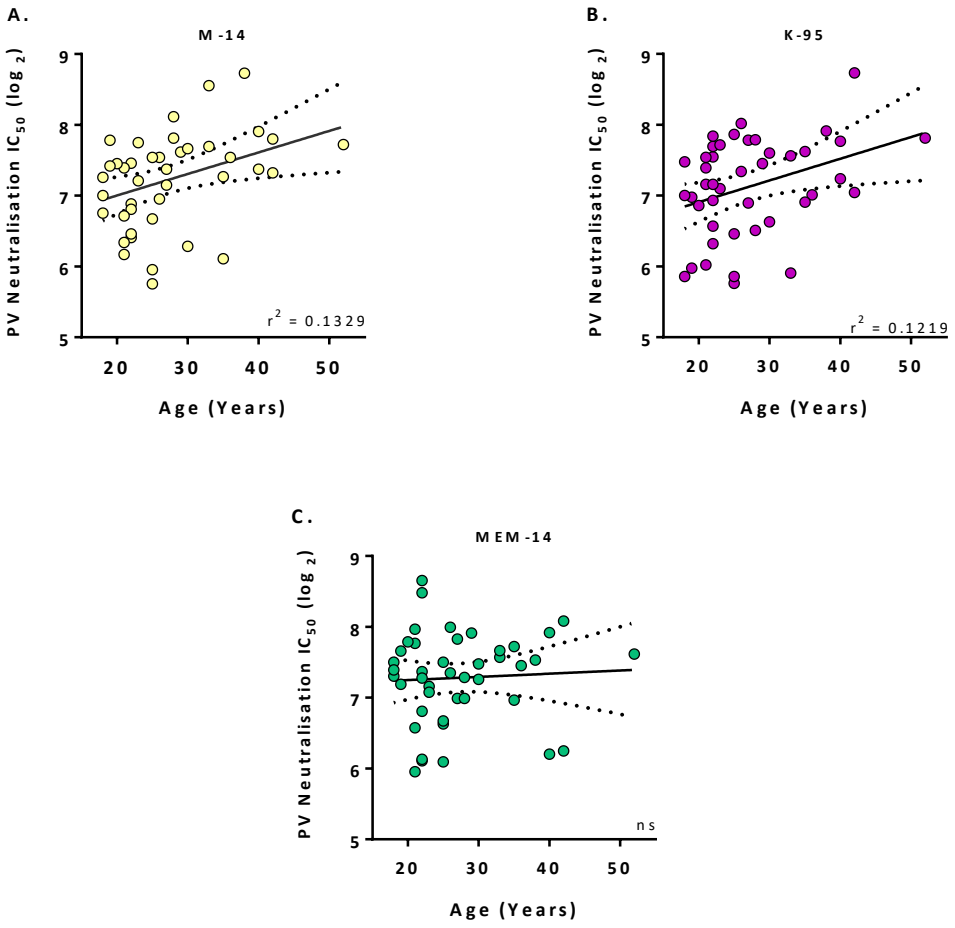


Figure 4.6 Effect of age on EBOV neutralising titres

Linear regression analysis was used to assess the extent to which EBOV NAb titres was dependent on age of the survivors. C) MEM-14 showed no statistical relationship between the two variables, however, age was observed to be a very weak predictor of NAb titres for A) M-14 r^2= 0.1329 and B) K-95, r^2= 0.1219.

4.2.4.3 EBOV Ab neutralisation potential distinguished by the body mass index (BMI) of cohort

The BMI is a measure that takes into account an individual's height and weight to work out if the person's weight is considered healthy. To work out BMI, an adult's weight (kg) is divided by their height (metres squared) which can be used to check a general weight status (Table 4.3) (NHS, 2016).

Table 4.3 Adult BMI ranges and corresponding weight status

BMI is a value obtained to evaluate if an individual's weight is considered healthy. BMI is calculated by dividing an adult's weight (kg) by their height (metres squared) (Adapted from NHS., 2016).

BMI	Weight Status
< 18.5	Underweight range
18.5 – 24.9	Healthy weight range
25 – 29.9	Overweight weight range
30 – 39.9	Obese range

Numerous epidemiological data suggests there is an association between body weight and rate of infection risk and also outcome of disease. A combination of malnutrition and poor hygiene standards in developing countries, may contribute to high infection rates (Dobner and Kaser, 2018). Likewise obesity is associated with compromised immune responses, thus emphasising the interaction between metabolic control and immune tolerance (Carbone et al., 2016). Several studies have indicated normal weight is linked with lowest infection risk as opposed to those who are underweight or obese (Dobner and Kaser, 2018). Research studying the effect of obesity as an independent risk factor for poor vaccine induced immune response in humans, found a positive correlation between the two variables in separate vaccine studies for hepatitis A (Van der Wielen et al., 2006) and B, rabies (Banga et al., 2014), influenza A/pH1N1 and tetanus (Painter et al., 2016). A previous study monitored the Ab responses to the 2009-2010 inactivated trivalent influenza vaccine (TIV) in a mixed BMI cohort including healthy, overweight and obese participants. It found that although there was an initial positive correlation between BMI and IgG Ab titres, 12 months post vaccination higher BMI was related to a greater decline in influenza Ab titres (Sheridan et al., 2012).

All donors from this cohort are over 18 years of age and classed as adults therefore the BMI of the whole cohort could be used for further evaluation. BMI was recorded at time of study recruitment. BMI was assessed to see if there were any trends with the neutralisation potential of CP. The majority of the cohort fell into the BMI range for healthy weight (18.5–24.9) with the remaining IC_{50} values falling into overweight (25-29.9) and obese (30-39.9) which were grouped together due to limited data points, allowing comparative analysis to be undertaken.

As not all data sets passed the normality test and there were slight variations in standard deviations, a Mann-Whitney U-test was used to evaluate potential differences between IC_{50} titres categorised as healthy and overweight/ obese within each virus strain, however, significant differences were not observed (Figure 4.7 A). Kruskal-Wallis test was also applied to the data, to observe if there were differences between the EBOV strains within each BMI group, this analysis also yielded non-significant differences in results. The neutralisation IC_{50} titres for each strain within each BMI category were compiled; healthy (n= 99) and overweight/ obese (n= 30) to assess if overall there were any trends between weight status and the neutralisation capacity of EBOV NAbs using a Mann-Whitney U test, however, no significant differences were observed (Figure 4.7 B). The results indicate there are no observable differences between the BMI of donors and the neutralisation capacity of EBOV NAbs within this cohort, as well as no observable differential effect caused by EBOV strains.

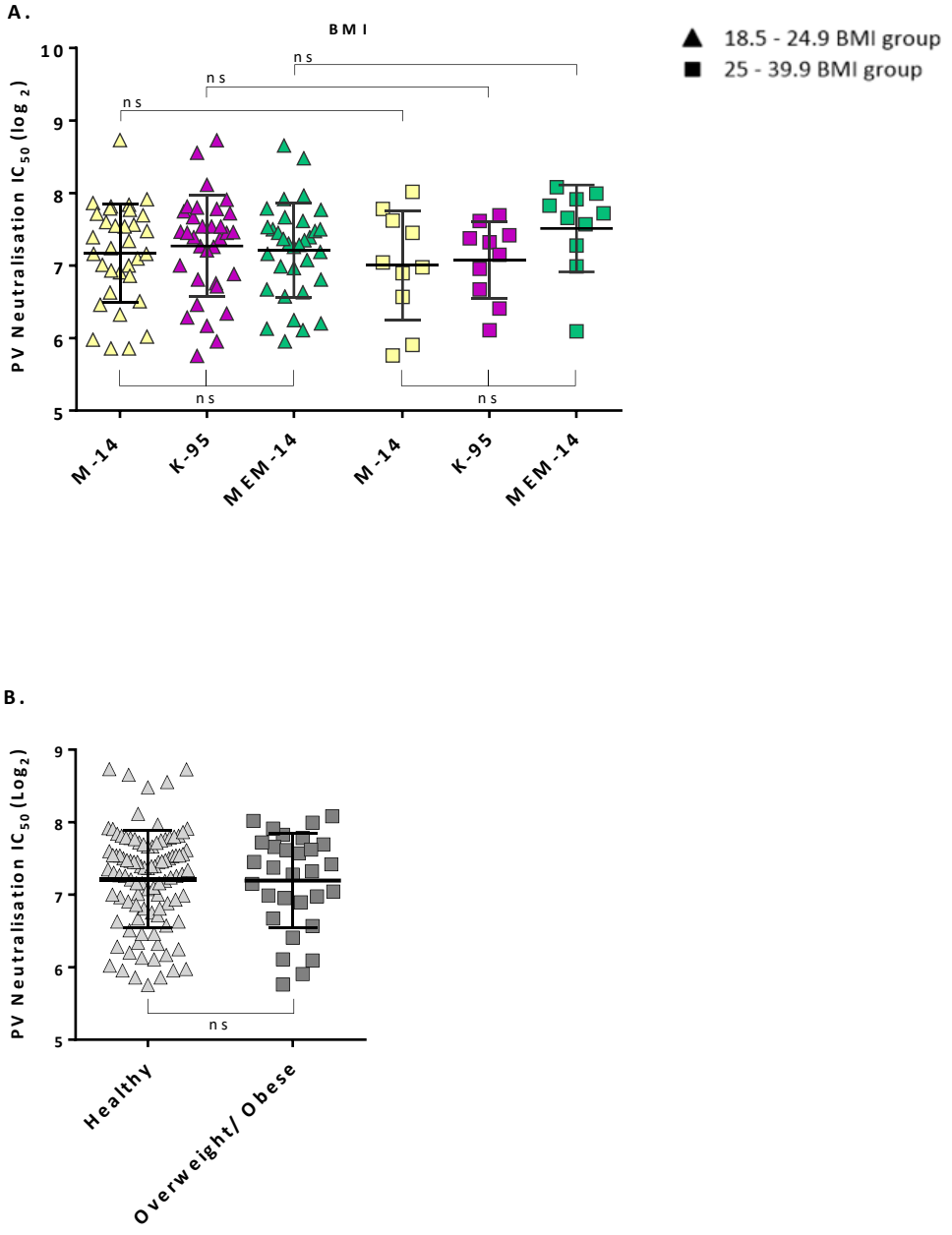


Figure 4.7 PV Neutralisation IC_{50} titres of EBOV strains distinguished by BMI of cohort

A) IC_{50} titres were grouped according to weight status; healthy 18.5-24.9 and overweight/ obese 25-39.9. A Mann-Whitney U test was used to assess differences between both BMI groups for a particular EBOV strain. Kruskal-Wallis test was applied to analyse differences between M-14, K-95 and MEM-14 EBOV strains within the BMI group. No statistical significance was observed for either test. B) The titres for the EBOV strains were compiled into healthy and overweight/ obese BMI groups and statistically analysed using a Mann-Whitney U test, results revealed no significant differences. Error bars represent mean with standard deviation.

4.2.4.4 EBOV Ab neutralisation potential distinguished by severity of disease of cohort

The effect of clinical severity of disease in relation to NAb production has been studied in patients infected with severe acute respiratory syndrome (SARS). It was found that patients exhibiting a more severe clinical course had earlier and higher Ab responses. Clinical severity was found to positively correlate with SARS NAbs and in some patients who experienced mild infection, detectable NAb responses did not develop (Ho et al., 2005). To see if there were any trends between EVD severity and EBOV neutralisation titres in CP, the IC_{50} values extracted from the neutralisation curves were categorised by EBOV strain and clinical severity; mild, moderate or severe as stated by the donor. As EVD severity was documented for less than half the cohort (n= 23) not all statistical analysis could be undertaken, including comparison between severity groups for each EBOV strain.

Analysis between the three EBOV strains within one symptom group was carried out using the Kruskal-Wallis test, however, no significant differences were observed (Figure 4.8 A). In order to compare if overall there were any associations between disease severity and neutralisation capacity of EBOV specific Abs, neutralisation titres of all three EBOV strains were compiled into symptom groups; mild (n= 24), moderate (n= 30) and severe (n= 15) and were scrutinised by a Kruskal-Wallis test which also revealed no statistically significant differences between the three symptom groups (Figure 4.8 B). Although not statistically significant a trend could be observed between NAb titres and EVD severity of donors within the cohort; a decrease in IC_{50} titres with increased EVD symptom severity was seen (Figure 4.8 B).

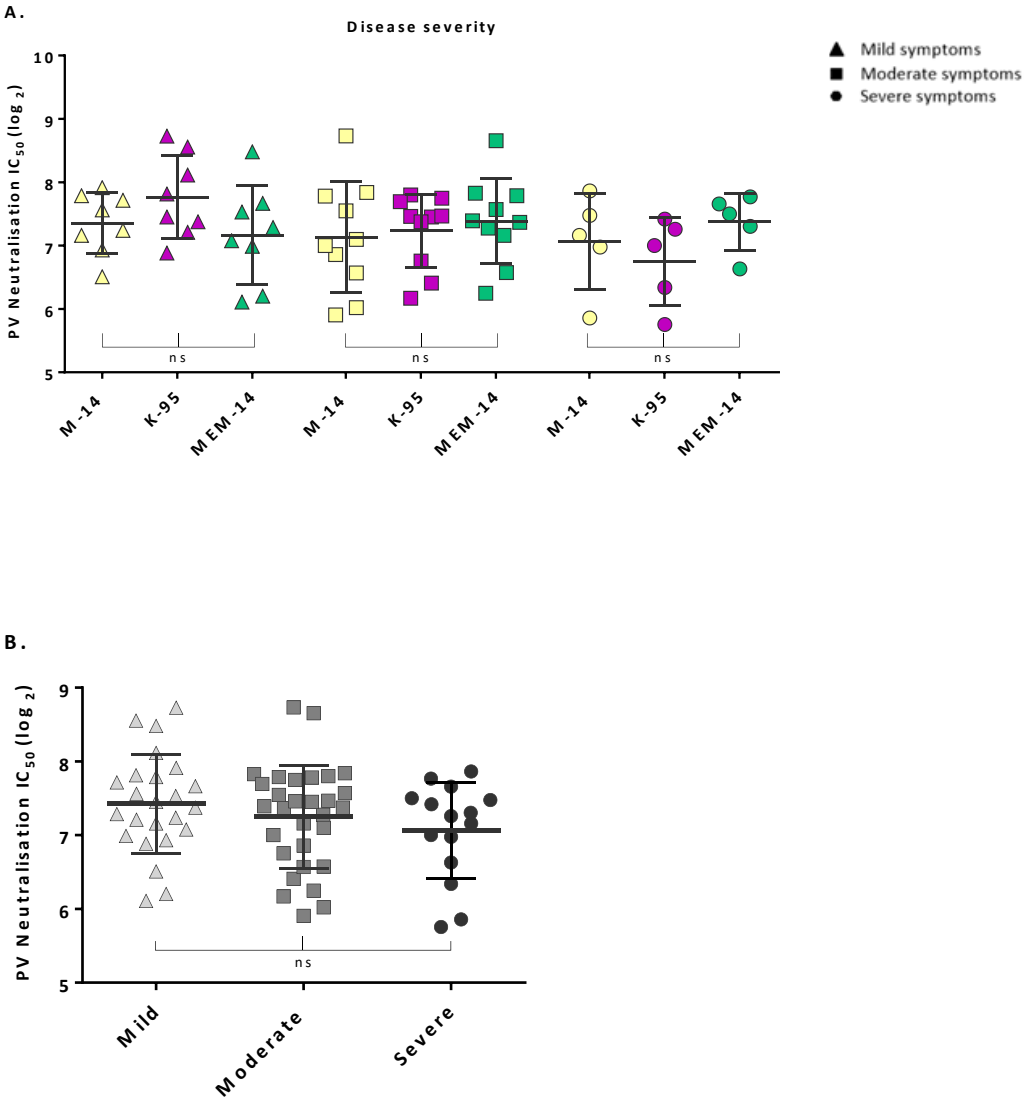


Figure 4.8 IC_{50} Neutralisation of EBOV strains distinguished by severity of disease of cohort

A) IC_{50} titres were grouped according to EBOV virus strain and disease severity; mild, moderate and severe as stated by the donor post-EVD. A Kruskal-Wallis test was applied to analyse differences between M-14, K-95 and MEM-14 EBOV strains within severity groups; statistically significant differences were not observed. B) The titres for the EBOV strains were compiled according to disease severity groups and was analysed with a Kruskal-Wallis test, results revealed no significant differences. Error bars represent mean with standard deviation.

4.2.5 EBOV NAb analysis according to sampling time

Donor demographics including gender, age, BMI as well as severity of disease did not show any strong significant associations with the anti-EBOV neutralisation capacity present in CP. Thus sampling time post convalescence was analysed as an independent parameter to investigate if any inferences could be made with regards to the previously observed wide ranging neutralisation potential seen in the anti-EBOV Abs present in CP (Figure 4.2) Recovery from EBOV is associated with long-lasting EBOV specific immunoglobulins (Sanchez-Lockhart et al., 2018), however, the repertoire of NAbs may increase later in the convalescent period so it could be expected that samples provided earlier in the recruitment period (closer to the convalescent period) would have higher neutralising titres than those donated much later post convalescence (Maruyama et al., 1999b).

To investigate whether this trend was apparent in our cohort, neutralisation IC_{50} titres were categorised according to the day the sample was taken post convalescence (convalescence standardised to day 0, when the donor tested double negative for Ebola). It can be seen that majority of the samples (n= 19) for A) M-14, C) K-95 and E) MEM-14 were donated between 200-300 DPC (Figure 4.9). A Kruskal-Wallis test was used to analyse the IC_{50} titres across the intervals, for each virus strain. The analysis revealed no significant differences of EBOV NAb titres between the four intervals; 0-100, 100-200, 200-300 and 300-400 (days) for the EBOV strains A) M-14 and C) K-95 (Figure 4.9). Although for E) MEM-14 a significant difference ($p= 0.0083$) was observed between the time the plasma sample was donated post convalescence and the neutralising titre of the EBOV NAb (Figure 4.9).

To determine if any overall trends could be inferred between the two variables; DPC and the capacity of EBOV-GP specific Abs to neutralise EBOV, IC_{50} titres were plotted against DPC for each virus strain B) M-14, D) K-95 and F) MEM-14) and a linear regression analysis was applied to the data, however, no statistical significance was observed (Figure 4.9).

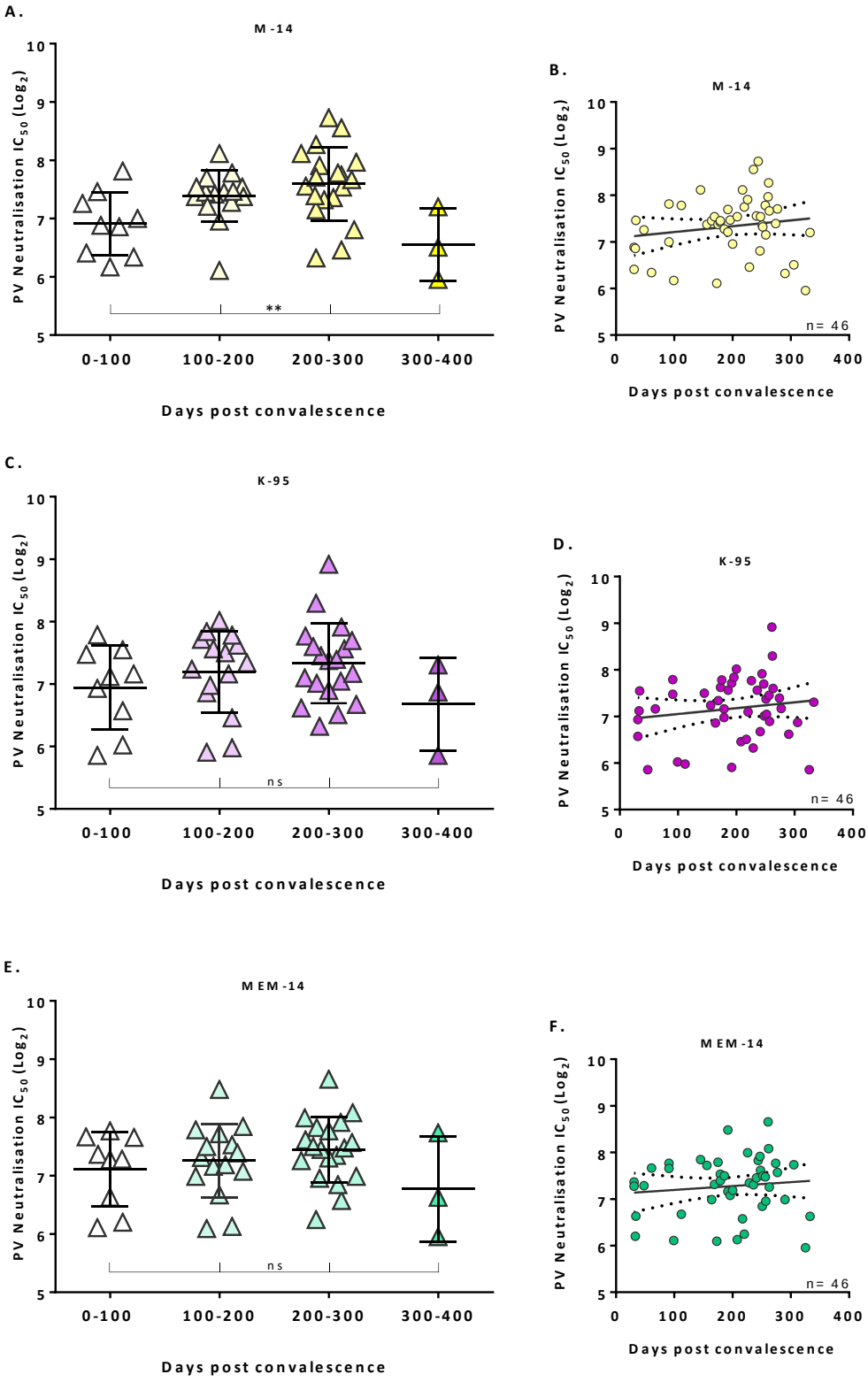


Figure 4.9 Neutralisation IC_{50} titres assessed against sampling time post convalescence

Neutralisation IC_{50} titres were grouped into intervals of days post convalescence (Day 0 standardised; donor tested double negative for EBOV). A Kruskal-Wallis test indicated, no statistically significant differences were observed for C) K-95 and E) MEM-14 however A) M-14 indicated a difference in neutralisation titres between the intervals (**P<0.01). Assessment of overall IC_{50} titres against days post convalescence yielded no statistically significant data with the linear regression analysis B), D) and F). Error bars represent mean with standard deviation.

4.3 Discussion

4.3.1 Independent risk factors on EBOV prevalence

4.3.1.1 Gender

The WHO have highlighted differences in gender-related roles that influenced exposure patterns, including: i) time spent at home and away from home, ii) obligation to care for the sick, iii) obligation to care for livestock, iv) healthcare access and v) scientific knowledge about treatment (Who., 2007) These gender associated roles have their own EBOV risk factors for transmission of virus. Known index cases of previous Ebola outbreaks have been traced to exposure of bush meat (Leroy et al., 2009), the hunting of bush meat as well as caring for livestock which are culturally associated with men within Africa (Nkangu et al., 2017). Likewise, women are usually considered the primary care givers including caring for family members who are sick, leading to exposure to the virus with infected individuals and bodily fluids (Nkangu et al., 2017). Cultural funeral practices put both men and woman at risk with some traditional rites which include touching, bathing and dressing the dead bodies. Carrying out these practices on someone who has died of Ebola exposes that individual to the virus (Nkangu et al., 2017), especially as dead bodies are known to secrete high viral loads (Brainard et al., 2016). The WHO indicated men and woman were possibly at equivalent risk of contracting Ebola in Africa through their gender-related roles in society. For this study early and multiple donated plasma samples were prioritised, gender was not a parameter used to select donors therefore the 2:1 ratio of males to females in this cohort was stochastic and the statistical analysis would take such numbers into account.

A WHO study has analysed the gender-specific epidemiologic patterns of 20,035 probable and confirmed cases of Ebola. Parameters such as incubation period, duration of hospitalisation, case fatality rates of EVD in Guinea, Liberia and Sierra Leone were considered to assess the differences between male and female persons (Gambacorti-Passerini et al., 2016). Overall it was found that although the frequency of exposure was higher for woman than men both sexes had a similar average risk of contracting the virus, the average interval symptom onset to hospitalisation, was around 0.5 days shorter for females compared to males and a noted significant difference was that females had a higher survival rate compared to male patients (Gambacorti-Passerini et al., 2016). A higher mortality rate in males (85%) to females (35%) was also concluded by a study conducted in the Moyamaba district, Sierra Leone (Haaskjold et al., 2016), consistent with the mortality finding; males (40%) and females (29%) in a study in Kerry Town, Sierra Leone (Hunt et al., 2015).

The reasons for the difference in survival rates is unknown but could be due to the immunosuppressive effects of androgens and immunostimulatory effect of estrogen (Haaskjold et al., 2016) and likewise a higher survival rate could be attributed to a robust early immune response to EBOV; equated with a higher production of EBOV specific NAbs. This finding however, was not reflected in our cohort, statistical analysis revealed no observable differences in EBOV specific neutralisation titres between the male and female cohort for each EBOV strain; M-14, K-95 and MEM-14 and this was further iterated when the compiled IC_{50} titres of the EBOV strains revealed no differences between male and female donors.

4.3.1.2 Age

A study which evaluated the effect of age on Ebola survival found that the average age of the people who died was 32 years (Interquartile range 23-43 years) and the age for those who survived was 26 years (interquartile range 15.25-34.75 years) (Li et al., 2016). There was no statistical difference in fatality rate between the 18-40 years age group compared to the over 40 age group, however, there was a statistically significant lower fatality rate for those under 18 years. Fatality rates could be attributed to an insufficient immune response therefore no differences between the lower and higher age groups in this cohort indicates age may not have been a variable affecting immune response.

It was observed in the cohort that for M-14 and MEM-14 there were no differences in NAb titres between the lower (18-26 years) and higher (27-52 years) age groups. This result mirrors that of the mentioned study which found no difference in fatality rate between the 18-40 years and over 40 age group. The similarity in NAb titres between the two age groups suggests that at least within the age group examined, 18-52 years, age is not a factor influencing the sufficient production of NAb titres for EBOV survival in EVD sufferers. This may also reflect the similarity in fatality rates observed by the study mentioned above. However, a statistical difference was observed between the lower and higher age group for K-95 ($P= 0.0036$) and in the linear regression analysis both K-95 ($p= 0.1219$) and M-14 ($p= 0.1329$) indicated that age could be a predictor of NAb titres as suggested by some studies (Schieffelin et al., 2014). Majority of the cohort were between the ages of 18-30; young adults possibly with stronger immune systems, this may explain why age was not seen as a

strong predictor of NAb titres within the cohort. Statistical significance may have been different with a larger, wide-ranging age group.

4.3.1.3 BMI

As yet there is no published data on the effect of BMI as an independent risk factor for Ebola outcome. Available data indicates individuals who are considered underweight or obese are at the highest risk of infections (Dobner and Kaser, 2018) also a positive correlation has been observed between obesity and a reduction in vaccine induced immune responses in several studies (Reviewed Painter *et al.*, 2016). Adipose tissue recognised as part of the endocrine system, consists of adipocytes and macrophages that through signalling molecules can induce a chronic state of inflammation potentially worsening immune complication in obesity (Painter et al., 2016).

It has been shown that a chronic inflammatory state can interfere with vaccine-induced immune response possibly due to poor responses being induced to antigens, which could be theorised to also occur in overweight/obese individuals suffering from EVD. A poor immune antigen response could be a result of reduced Ab responses including anti-EBOV NAb induction. A difference in neutralisation titres between healthy and overweight/ obese individuals was not observed in the cohort however, with larger sample numbers the three weight status; healthy, overweight and obese could have been considered individually revealing more comparative analyses.

4.3.1.4 Severity

In this study severity of disease was retrospective and defined by the donors themselves during the recruitment process; how the donors felt their clinical manifestations at time of disease were compared to other patients. There is no published data on severity of disease and NAb titres in EBOV survivors however, research comparing the clinical presentations of survivors and non-survivors, found that higher viral load, severe confusion, abdominal pain, vomiting and conjunctivitis (severe symptoms) was associated with poor prognosis in EVD patients (Ji et al., 2016). Although results were not statistically significant possibly due to insufficient data, an inverse trend was observed; with increased symptom levels there was a decrease in the capacity of EBOV-GP specific Abs to neutralise EBOV.

It could be theorised that the host's early immune response was impeded, thereby impairing a vigorous mounted humoral response. Consequently, the EVD patient would suffer from symptoms associated with severe EVD as described as well as reduced NAb titres as seen in our results. More data points; either the category of symptoms from the survivor's perspective or the clinical symptoms from the view of the clinician would need to be included to gain more informative or statistically relevant data on how EVD severity effects NAb titres.

4.3.2 Overall comparison of the three EBOV PV strains

IC_{50} is commonly used to establish clinical expectations of NAb activity from *in vitro* experimental results and for the identification of NAbs with high potential for advancement into clinical trials (Webb et al., 2015). In a prophylactic vaccine setting, where the multiplicity of infection is relatively low the neutralisation IC_{50} titre may be sufficient, however, to be utilised as an effective therapeutic dose in the presence of multiple logs of virus (Webb et al., 2015) a higher neutralisation titre (IC_{70}-IC_{90}) would be desirable. In further support of these results shown here, neutralisation IC_{70} titres of the three PV strains were also extracted from neutralisation curves and compared, no significant differences were observed (See appendix).

Neutralisation IC_{50} titres and IC_{70} were used to assess CP inhibition of EBOV PV strains; K-95, M-14 and MEM-14, overall no differences were observed in the capacity of 2014 CP to inhibit the three EBOV PV strains; K-95, M-14 and MEM-14. These results would imply that vaccination incorporating antigens of either the 1995 Kikwit or 2013 Makona EBOV strains would be able to elicit NAbs in the recipient that would be cross-reactive with either strain. Previous studies using VSV expressing the EBOV GP grown in the presence of anti-GP Abs observed that a single amino acid substitution conferred viral resistance to the Abs (Kajihara et al., 2013). GP mutational changes have also been observed to impact immune responses to EBOV; substitutions at N-linked glycosylation sites can alter antigenicity and immunogenicity and in some cases preventing binding to the KZ52 Ab (Dowling et al., 2006; Lennemann et al., 2015; Miller et al., 2016). As no overall differences were observed in the potency of 2014 CP to neutralise the EBOV strain bearing GP mutations; MEM-14 compared to M-14, this indicates that the four amino acid differences seen between the strains in the GP_1 head, mucin like domain and the fusion loop were possibly not prominent neutralising epitopes recognised by the CP NAbs.

The V920 vaccine has been shown to provide long-term immunity in the vaccinated cohorts; it is to date the most promising filovirus vaccine. However, it was found that NAb titres within the vaccinated cohorts were low and declined quickly (Huttner et al., 2018). Although NAbs are not solely responsible in EBOV survival they have been documented to play a key role (Payne, 2017). Results in this chapter demonstrated that NAb titres varied between individuals and CP samples with more potent neutralisation potential resulted in greater inhibition of EBOV and vice versa. This indicates that although Ab titres were maintained in the vaccinated cohort the reduction in NAbs could be detrimental to the overall protective effect of the vaccine. Therefore, the vaccination induced immunity to EBOV should be assessed for a longer period in vaccinated individuals.

4.3.3 Sampling time on EBOV NAb

Post disease onset, antigen-specific Abs generally persist in circulation for a long period of time (Moody et al., 2011). Although Abs have been found to persist in EBOV survivors for long periods; IgG Abs have been detected in survivors up to 40 years post disease onset (Rimoin et al., 2018), it is not known whether the resurgence of EBOV documented in a number of Ebola survivors (Diallo et al., 2016) has an association with a declining adaptive immune response, specifically EBOV NAbs. Therefore, plasma donated at longer periods post convalescence may have less neutralising capability then plasma donated after a shorter period. The overall statistical analysis of the results revealed no significant trends between NAb titres and the time the sample was donated post convalescence, for M-14, K-95 and MEM-14 EBOV strains. Therefore, as no differential neutralisation IC_{50} titres were observed during the plasma donation period, this cannot be attributed as the cause of the wide ranging neutralisation capacities of the donor CP observed for the three virus strains. To explore this further and investigate if the varied neutralisation potential was attributed to individual donor immune responses a longitudinal study was conducted whereby the analysis of multiple samples of a donor would illustrate individual neutralisation profiles of the survivors.

4.3.4 Chapter conclusions

The results presented in this chapter illustrated that the EBOV PV neutralisation assay we developed, was utilised for the successful evaluation of Ebola survivors CP NAbs. The results revealed a wide-range of CP neutralisation potential between the donor samples which was not attributed to the time post convalescence the sample was donated. Utilising the limited

clinical information available for this cohort our analysis revealed that neither gender, age, BMI or disease severity was a strong significant predictor of CP NAb titre. A positive linear regression analysis observed for age and CP NAbs for M-14 and K-95 indicated that age could impact NAb titres, however, it is possible a larger data set was needed for this analysis. Although, not significant an inverse trend was observed for disease severity; NAbs decreased with increased severity, this could potentially be attributed to an impairment of the immune system possibly due to high viral loads. This is the first study to assess NAbs in the context of donor clinical parameters, NAbs have a role in preventing, reducing and clearing infection (Morris and Moody, 2016), understanding how clinical parameters can influence NAb titres could lead to further understanding to individuals who are at high risk to EBOV, thus this is an area that should be built upon.

5 Longitudinal analysis of neutralising Abs from convalescent plasma of Ebola survivors

5.1 Introduction

5.1.1 Longitudinal Study

A longitudinal study uses repeated procedures to follow specific individuals over a period of time. The data acquired from individuals within a predefined cohort can be quantitative and/ or qualitative without external influences being applied. Therefore statistical analysis can be applied to analyse changes over time for the group as a whole or for specific and selected individuals (García-Peña et al., 2015).

Advantages of a longitudinal study that apply to the analysis here include (García-Peña et al., 2015):

- Tracking changes over time of particular individuals within a cohort or following the cohort as a whole.
- Ability to identify and relate events to particular exposures and define the exposures with regards to presence, timing and chronicity.

5.1.2 Anti-EBOV Abs over time

Due to there being no well-established correlates of protection, the longevity of immune Ab responses induced by EBOV or EBOV vaccines has largely remained unknown, however, data in this field has recently come to light (Bornholdt and Bradfute, 2018). The study of the V920 vaccine which expressed the Zaire EBOV GP, found that human participants from multiple sites across Africa and Europe retained quantifiable EBOV specific Ab responses for 1-2 years after inoculation. Ab responses were maintained for both groups of participants who received a low (300 000 plaque forming units [pfu]) or a high (10-50 million pfu) single dose of the vaccine. Interestingly, although overall Ab titres were sustained over this period EBOV NAb titres were low and declined within 6 months in both vaccine groups in a subset of participants, therefore, the long-term immunity provided by the Ab titres remains unknown and requires continued assessment (Huttner et al., 2018). There is little documentation of long-term natural EBOV Ab titres in Ebola survivors. It has been demonstrated that *Filoviridae* specific Abs are maintained up to 11 years, post-infection in survivors. One study found two

survivors of the 1995 Kikwit DRC outbreak retained memory B cells and Abs specific to EBOV, as well as mAbs isolated from one survivor showing potent neutralising activity against EBOV GP more than a decade after infection (Corti et al., 2016). Another study assessing Ab levels to EBOV GP, NP and VP40 in Ebola (*Sudan* and *Bundibugyo ebolavirus*) or Marburg survivors found that there were persistent levels of Abs to all three EBOV antigens 14 years after infection, with Abs to GP being the most specific (Natesan et al., 2016).

A study analysing anti-EBOV Ab persistence and neutralisation capacity in EBOV survivors was performed 40 years post-infection; the longest documentation of anti-Ebola serological responses and neutralising capacity within any cohort of survivors (Rimoin et al., 2018). The 14 survivors were from the 1976 Yambuku DRC outbreak and where serum samples were examined for reactivity to EBOV GP, EBOV NP and EBOV VP40. Plasma reactivity to GP and NP were observed in the majority of the cohort whilst immunoreactivity to VP40 was limited to a few survivors. PRNT was used to assess neutralising Ab responses of serum samples with replication competent EBOV and where it was observed that four survivors had anti-EBOV neutralising titres, with the two survivors having the highest neutralisation capacity also having reactivity to VP40. The study showed survivors harboured Abs to EBOV viral antigens and that a subset of individuals were still capable of neutralising RCE, 40 years post infection. This indicates EBOV is able to elicit a life-long humoral immune response and presumably provide protection against infection (Rimoin et al., 2018).

5.1.3 Assays measuring anti-EBOV Abs in CP

As previously discussed the most extensively employed assays used to assess CP NAbs are PV assays for their ability to measure NAbs with high throughput, ELISAs for their capacity to yield quantitative data and PRNT, which is the assay most reflective of biological responses. These assays all consider the conformational epitope and glycosylation of EBOV.

5.1.4 Summation

As described in Chapter 4 (4.2.1), Ebola survivors were recruited for plasma donations as part of the study "Convalescent plasma for early Ebola virus disease in Sierra Leone". A sub-population of donors were selected for our study. The overall aim of this study was to evaluate the efficacy with which NAbs present in CP of Ebola survivor's, inhibited the viral entry of EBOV PV. IC_{50} titres acquired from neutralisation assays with CP from our cohort revealed an expansive neutralisation capacity of EBOV PV between donors (results shown in

4.2.2.1). A cross-sectional assessment of select IC_{50} titres from the neutralisation assays revealed a wide-ranging neutralisation potential of EBOV CP from the cohort could not be statistically attributed to sex, age, BMI, disease severity or the time in which the CP sample was donated post convalescence (standardised as the day that the survivor was confirmed double negative for EBOV RNA in blood).

5.1.5 Aims of this Chapter

A longitudinal analysis of the CP samples within our Ebola survivor cohort was undertaken to expand on the results of chapter 4, in order to discern the differential inhibition potential of CP observed between donors. By including PV NAb titres of multiple CP samples donated from individuals within the cohort, we sought to gain an insight into:

1. CP NAb potency over time
2. NAb trends within longitudinal donor CP samples
3. The efficacy of NAb titres measured with the PV assay compared with a PRNT using RCE
4. The correlation between NAb titres measured with the PV assay and the quantity of total anti-EBOV Abs to EBOV GP measured with an EIA
5. An unusual NAb profile of a donor in the cohort which was methodically investigated by:
    - Donor Enquiry
    - Assessing the PV NAb trend against titres of Abs targeting EBOV NP, VP40 and GP measured with EIAs using RCE
6. The differences of CP NAb titres between the three PV EBOV strains; M-14, K-95 and MEM-14
7. The growth and decline rate of CP NAbs through mathematical analysis

5.2 Results

5.2.1 Longitudinal study Cohort

Donors in the cohort who donated three samples or more during the recruitment period were utilised in this analysis. Donors included in the longitudinal study along with the corresponding CP samples donated are listed in Table 5.1. Donor ID listed in bold were samples utilised in the cross-sectional analysis in Chapter 4. The shortest longitudinal sampling period for an individual donor was over 79 days by IGH_EBOV CP Donor_007 whilst IGH_EBOV CP Donor_021 provided samples over a 254 day period and also provided the last sample of the cohort post convalescence; 505 days. Table 5.2 provides a summary of the CP samples included in the study as well as total sample numbers available for use, when and how many samples were donated post convalescence by a donor, and the number of samples corresponding to other assays including DABA and the RCE PRNT assay which are discussed further in this chapter.

Table 5.1 Donor CP samples used in this study

Bold Donor ID indicates CP sample used in the cross-sectional study. Convalescence was standardised; day 0= day donor tested negative for EBOV.

Donor ID	Days post convalescence
IGH_EBOV CP Donor_001	333
IGH_EBOV CP Donor_002_A	172
IGH_EBOV CP Donor_002_B	248
IGH_EBOV CP Donor_002_C	290
IGH_EBOV CP Donor_003_A	241
IGH_EBOV CP Donor_003_B	283
IGH_EBOV CP Donor_003_C	380
IGH_EBOV CP Donor_003_D	431
IGH_EBOV CP Donor_004_A	191
IGH_EBOV CP Donor_004_B	263
IGH_EBOV CP Donor_005	256
IGH_EBOV CP Donor_006_A	158
IGH_EBOV CP Donor_006_B	164
IGH_EBOV CP Donor_006_C	247
IGH_EBOV CP Donor_007_A	172
IGH_EBOV CP Donor_007_B	173
IGH_EBOV CP Donor_007_C	251
IGH_EBOV CP Donor_008_A	228
IGH_EBOV CP Donor_008_B	249
IGH_EBOV CP Donor_009	141
IGH_EBOV CP Donor_010	138
IGH_EBOV CP Donor_011	257
IGH_EBOV CP Donor_012	290
IGH_EBOV CP Donor_013	216
IGH_EBOV CP Donor_014	169
IGH_EBOV CP Donor_015	229

Donor ID	Days post convalescence
IGH_EBOV CP Donor_016	208
IGH_EBOV CP Donor_017	252
IGH_EBOV CP Donor_018_A	262
IGH_EBOV CP Donor_018_B	330
IGH_EBOV CP Donor_018_C	368
IGH_EBOV CP Donor_018_D	437
IGH_EBOV CP Donor_019_A	211
IGH_EBOV CP Donor_019_B	226
IGH_EBOV CP Donor_019_C	259
IGH_EBOV CP Donor_019_D	365
IGH_EBOV CP Donor_019_E	402
IGH_EBOV CP Donor_019_F	417
IGH_EBOV CP Donor_019_G	465
IGH_EBOV CP Donor_020_A	218
IGH_EBOV CP Donor_020_B	219
IGH_EBOV CP Donor_020_C	220
IGH_EBOV CP Donor_020_D	226
IGH_EBOV CP Donor_020_E	305
IGH_EBOV CP Donor_021_A	218
IGH_EBOV CP Donor_021_B	219
IGH_EBOV CP Donor_021_C	226
IGH_EBOV CP Donor_021_D	241
IGH_EBOV CP Donor_021_E	274
IGH_EBOV CP Donor_021_F	403
IGH_EBOV CP Donor_021_G	430
IGH_EBOV CP Donor_021_H	467

Donor ID	Days post convalescence
IGH_EBOV CP Donor_021_I	484
IGH_EBOV CP Donor_021_J	505
IGH_EBOV CP Donor_022	325
IGH_EBOV CP Donor_023	115
IGH_EBOV CP Donor_024	112
IGH_EBOV CP Donor_025	200
IGH_EBOV CP Donor_026_A	85
IGH_EBOV CP Donor_026_B	89
IGH_EBOV CP Donor_026_C	145
IGH_EBOV CP Donor_026_D	199
IGH_EBOV CP Donor_026_E	271
IGH_EBOV CP Donor_027	173
IGH_EBOV CP Donor_028	156
IGH_EBOV CP Donor_029	33
IGH_EBOV CP Donor_030_A	31
IGH_EBOV CP Donor_030_B	145
IGH_EBOV CP Donor_030_C	166
IGH_EBOV CP Donor_031	34
IGH_EBOV CP Donor_032	31
IGH_EBOV CP Donor_033	31
IGH_EBOV CP Donor_034	99
IGH_EBOV CP Donor_035	217
IGH_EBOV CP Donor_036	48
IGH_EBOV CP Donor_037	236
IGH_EBOV CP Donor_038	61
IGH_EBOV CP Donor_039	91

Donor ID	Days post convalescence
IGH_EBOV CP Donor_040	164
IGH_EBOV CP Donor_041	175
IGH_EBOV CP Donor_042	244
IGH_EBOV CP Donor_043	179
IGH_EBOV CP Donor_044	91
IGH_EBOV CP Donor_045_A	186
IGH_EBOV CP Donor_045_B	242
IGH_EBOV CP Donor_045_C	256
IGH_EBOV CP Donor_045_D	270
IGH_EBOV CP Donor_045_E	293
IGH_EBOV CP Donor_045_F	318
IGH_EBOV CP Donor_045_G	340
IGH_EBOV CP Donor_046	220
IGH_EBOV CP Donor_047	192
IGH_EBOV CP Donor_048	196
IGH_EBOV CP Donor_049_A	261
IGH_EBOV CP Donor_049_B	262
IGH_EBOV CP Donor_049_C	313
IGH_EBOV CP Donor_049_D	326
IGH_EBOV CP Donor_049_E	398
IGH_EBOV CP Donor_050	179
IGH_EBOV CP Donor_051	192
IGH_EBOV CP Donor_052	277

Table 5.2 Distribution of convalescent plasma samples donated from the cohort

Details of CP samples used in this study.

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Cohort details	Intervals	Total (n)
Cohort donors		52
Total number of CP samples available for use		101
Number of CP samples (Intervals) provided by a donor (n)	1	38
	2-4	8
	5-7	5
	10	1
CP donation post convalescence for all samples donated (days)	< 100	12
	100-199	25
	200-299	42
	300-399	12
	≥ 400	10
Total number of CP samples with a corresponding DABA value		99
Total number of CP samples with a corresponding replicaton competent EBOV neutralisation assay value		26

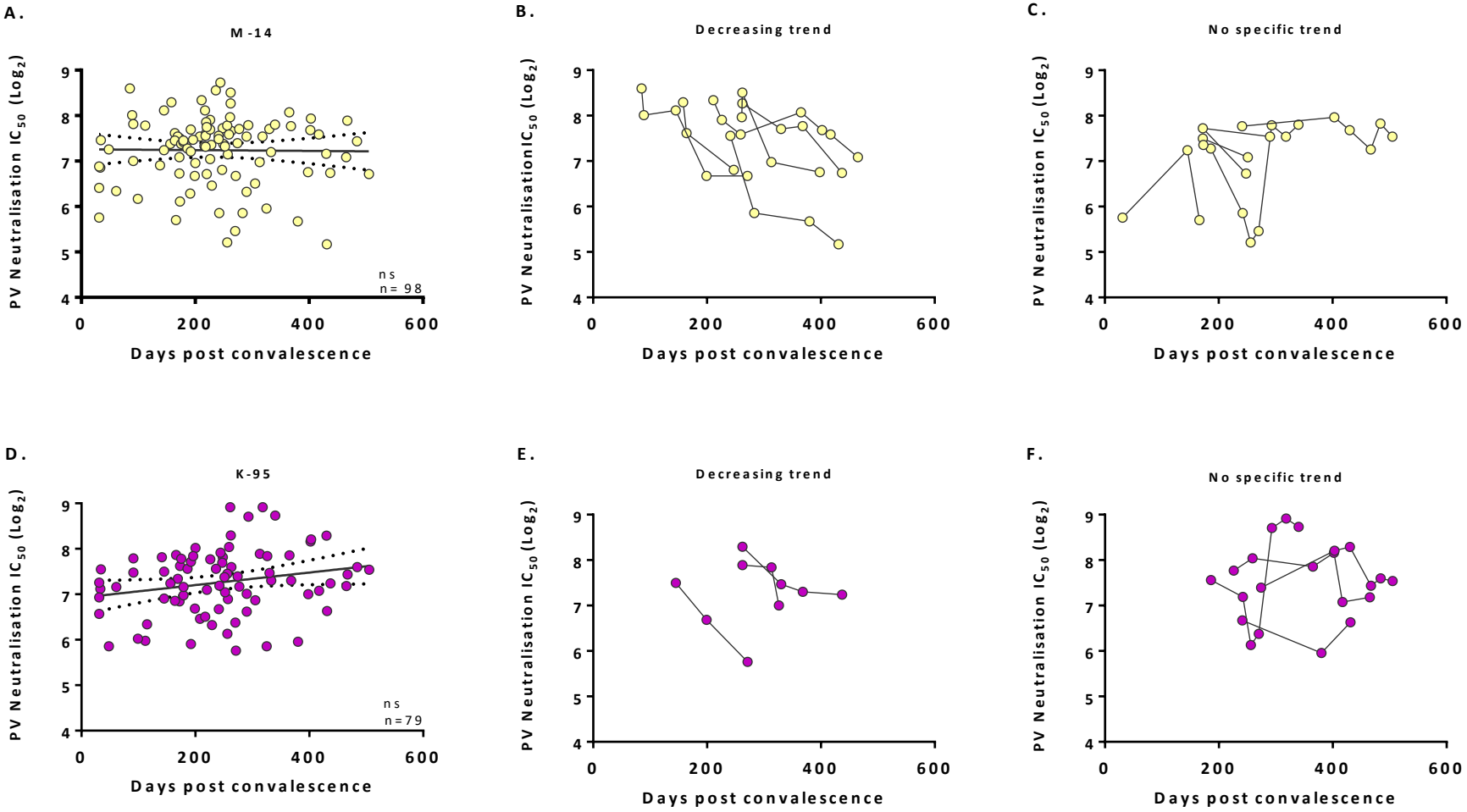
5.2.2 EBOV longitudinal NAb analysis according to Days post convalescence

It was previously concluded that within the CP samples used in the cross-sectional study (4.3.5) there were no statistically significant relationships determined between EBOV CP NAb titres and the time at which the samples were donated; days post convalescence (day 0, standardised as the day the donor was confirmed double negative for EBOV RNA by qPCR). Thus, indicating that donation time post convalescence was not a factor influencing the wide range of CP neutralisation potential observed previously.

In this analysis NAb titres of all CP samples included in the longitudinal cohort were assessed against days post convalescence for each virus strain with the view that additional samples included in the analysis could be more indicative of how Ab responses may change over time. It was observed, that the three EBOV strains A) M-14; the 2013 Guinea epidemic strain, D) K-95; the 1995 Kikwit strain and G) MEM-14; the 2013 Guinea strain comprising of mutations from early in the epidemic, all showed non-significant relationships (Figure 5.1). Therefore, this suggests at the population level there was no change in NAb titres indicating that titres

were stable over time or that the longitudinal assessment period was not long enough to measure significant changes.

When neutralisation IC_{50} titres from donors of the longitudinal cohort were scrutinised individually it could be seen that there were two broad groups that could be used to categorise the responses. Donors whose NAb titres generally: decreased (Figure 5.1. B, E and H) and donors whose titres which didn't show a distinctive drift towards an increase or decrease (Figure 5.1. C, F and I). The assignment of donors into the two groups was based on their profiles, as seen in (Figure 5.1. B, C, E, F, H and I), a "snapshot" of the neutralising profile for the respective donors within the given time frame. Based on the majority of donors falling into the group showing a decreasing trend, it could be hypothesised that it is likely all donors would eventually show a decreasing trend, however, more subsequent CP samples would have been needed to have studied this. Nevertheless, the "snapshot" of longitudinal samples provides an insight into NAb titres of individual convalescent Ebola survivors. It can be seen that donors categorised into the same group do not show exactly the same trends. In the group showing donors with a decreasing trend some decline over a longer period whilst others decreased quickly. Some donor's profiles showed a decrease but had NAb titres that still remained high whilst others demonstrated a steep decline in titres. Within the two groups, some donors can be seen to have a fluctuating profile with slow or quick, increases or decreases in NAb titres from one CP sample to the next. This result showed that EBOV NAb titres of convalescent Ebola donors can vary immensely between individuals.


A.
M-14
PV Neutralisation IC50 (Log2)
ns
n= 98
Days post convalescence
B.
Decreasing trend
PV Neutralisation IC50 (Log2)
Days post convalescence
C.
No specific trend
PV Neutralisation IC50 (Log2)
Days post convalescence
D.
K-95
PV Neutralisation IC50 (Log2)
ns
n=79
Days post convalescence
E.
Decreasing trend
PV Neutralisation IC50 (Log2)
Days post convalescence
F.
No specific trend
PV Neutralisation IC50 (Log2)
Days post convalescence

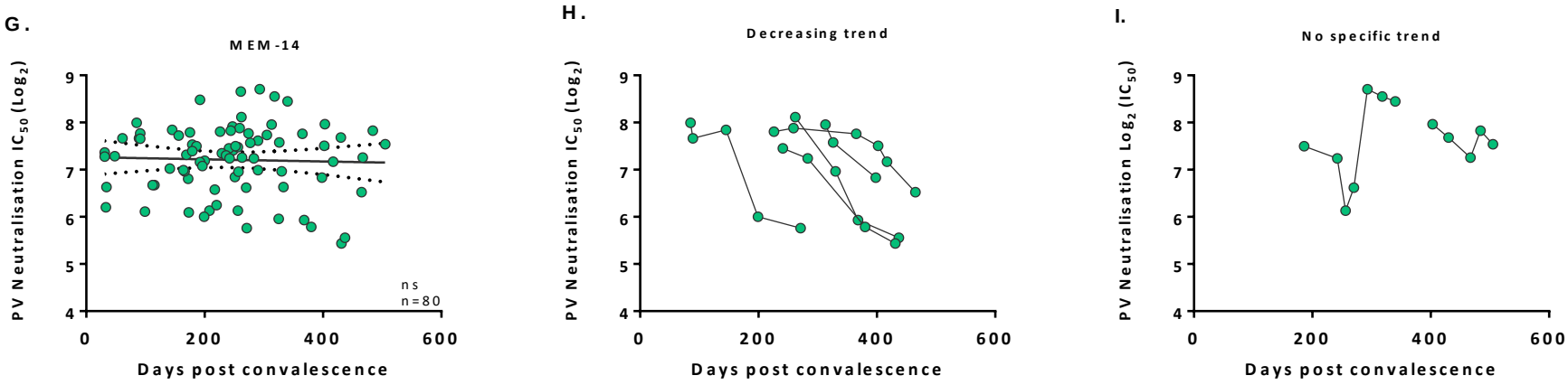


Figure 5.1 Longitudinal Neutralisation IC_{50} titres assessed against days post convalescence

Neutralisation IC_{50} titres of CP samples included in the longitudinal study were evaluated against days post convalescence (Day 0 standardised; donor tested double negative for EBOV) with a linear regression analysis for A) M-14, D) K-95 and G) MEM-14 which showed non-significant differences. Neutralising titres of longitudinal donors were evaluated individually and could be broadly categorised into two groups; NAb titres which decreased B) M-14, E) K-95 and H) MEM-14 and those with no distinctive trend C) M-14, F) K-95 and I) MEM-14.

5.2.3 EBOV PV NAbs evaluated against replication competent NAbs

The RCE PRNT is considered the gold standard assay for evaluating NAbs in sera or plasma as they measure a biological response with a natural antigen (Wilkinson et al., 2017). Due to the highly pathogenic nature of EBOV, its use is restricted to BSL-4 laboratories with stringent lab practices making high experimental throughput difficult (Mather et al., 2013).

Recently replication competent EBOV has been documented for use in the assessment of EBOV CP NAb titres, however, this was a study to establish an International Reference Reagent for the comparison and standardisation of EBOV serological assays (previously discussed in 3.2.5.2). The uniqueness of our study was the use of replication competent EBOV to assess the neutralisation potential of longitudinal samples of EBOV CP NAbs within a larger cohort of Ebola survivors. Due to time and expense constraints the samples utilised in the PRNT was limited to longitudinal CP samples donated which exhibited prominent neutralisation trends, including IGH_EBOV CP Donor_018, 019, 021, 045 and 049.

Both the PRNT and PV neutralisation IC_{50} titres obtained with the previous mentioned donor samples were first checked to see if the data followed a Gaussian distribution with a D'Agostino & Pearson omnibus normality test. The replication competent EBOV, K-95 and MEM-14 data all passed the normality test and therefore Pearson Correlation Coefficient (r) was applied to the data set whereas M-14 did not pass normality, therefore, a non-parametric Spearmann Correlation was used to assess the data. A positive association between the two data sets was observed and where a correlation coefficient (r) can range from +1 to -1 indicating a positive to negative association respectively between two variables. The r values and statistical significance (p values) of the correlation were as follows: Figure 5.2 A) 0.68, $p= 0.0003$, B) 0.84, $p<0.0001$ and C) 0.70, $p<0.0001$. The positive r values indicate the two variables move in the same direction; as one variable increases the other also increases and vice versa indicating a positive association whilst the p value lower than the alpha value of 0.05 indicates that the association is not likely due to chance.

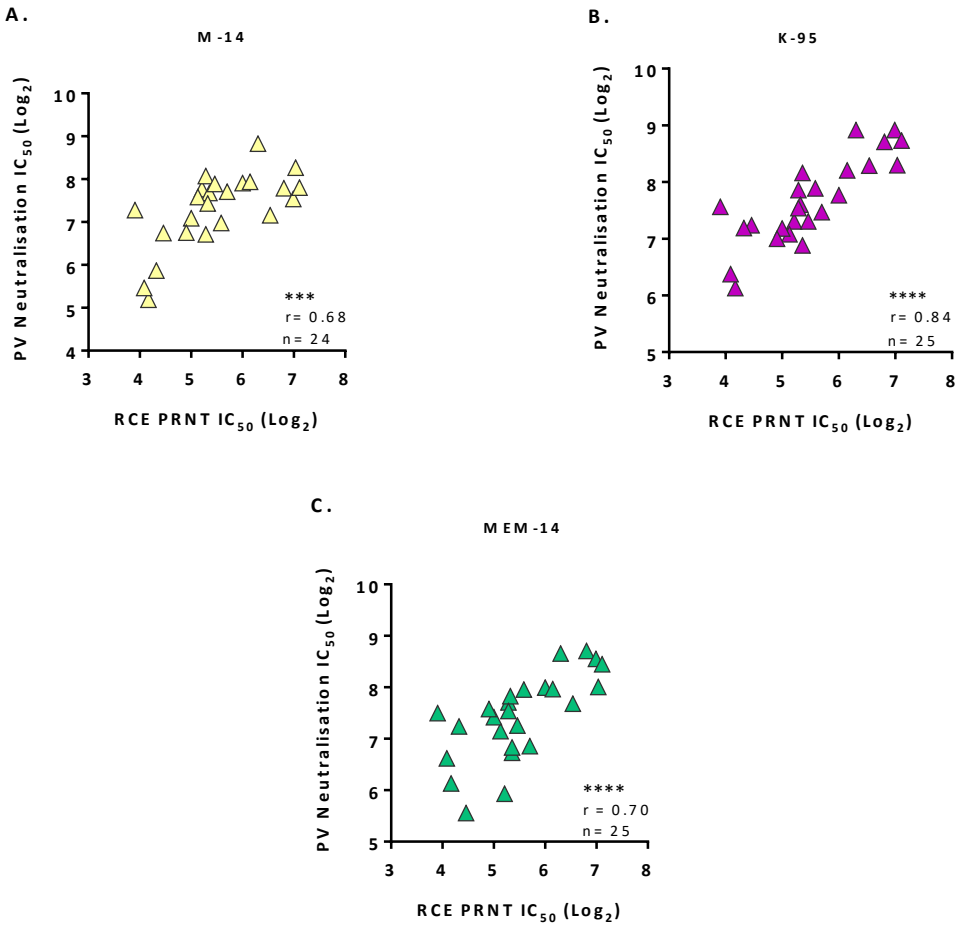


Figure 5.2 Correlation between EBOV PV neutralisation assay and EBOV replication competent PRNT assay

Neutralisation IC_{50} titres measured with the EBOV PV neutralisation assay were observed to have a positive association A) M-14 r= 0.68, B) K-95 r= 0.84 and C) MEM-14 r= 0.70 with the replication competent EBOV neutralisation titres measured by the PRNT assay. Correlation coefficients (r) statistics was applied to the data after passing D'Agostino & Pearson omnibus normality test. A non-parametric Spearman Correlation was applied to A) M-14 as a normality test was not passed. Statistical significant correlations were observed for A) M-14 (***P<0.001) and B) K-95 and C) MEM-14 (****p<0.0001).

5.2.4 Total Abs versus NAbs to EBOV GP

ELISAs utilised for the quantitative measurement of Abs present in plasma/ serum of donors is advantageous due to high throughput and rapid screening (Mather et al., 2013). ELISAs do not specifically quantify NAbs to EBOV but the total Abs targeting the antigen within the sample. None the less, a quantitative measurement of total Abs to EBOV GP permits comparisons with the NAbs titres measured by the PV neutralisation assay in order to deduce any statistical associations between the wide ranging-trends observed in CP with the PV assay.

An Enzyme Immune Assay (EIA); Double Antigen Bridging Assay (DABA) was developed as part of the "Convalescent plasma for early Ebola virus disease in Sierra Leone" study, headed by Tedder *et al.*, 2018. DABA was developed for the sensitive detection and quantification of anti-EBOV Abs present in human serum or plasma to the Zaire EBOV GP; the values attributed to the CP samples were made available for use in this study.

To assess if the differential NAb titres measured by the EBOV PV assay was reflected in the amount of anti-EBOV GP Abs present in the CP sample quantified by DABA, a correlation analysis was conducted between the two data sets; neutralisation IC_{50} titres and anti-EBOV GP Ab titres, to determine if there were any statistical associations. D'Agostino & Pearson omnibus normality test was used to assess if IC_{50} and DABA quantities followed a population with a Gaussian distribution; the cut off p value used was 0.05. IC_{50} titres of EBOV strain K-95, MEM-14 and DABA values passed the normality test, therefore a Pearson Correlation Coefficient was used to assess associations. However, M-14 did not pass the normality test ($p= 0.0136$) hence a non-parametric Spearman Correlation was used for analysis. Results indicated a statistically significant association between the two variables; neutralisation IC_{50} titres and DABA quantified anti-EBOV GP Abs for each of the three PV strains. The statistical analysis was as follows: A) M-14 $r= 0.46$, B) K-95 $r= 0.63$ and C) M-14 $r= 0.52$, all three EBOV strains showed a significant correlation ($p<0.0001$) (Figure 5.1).

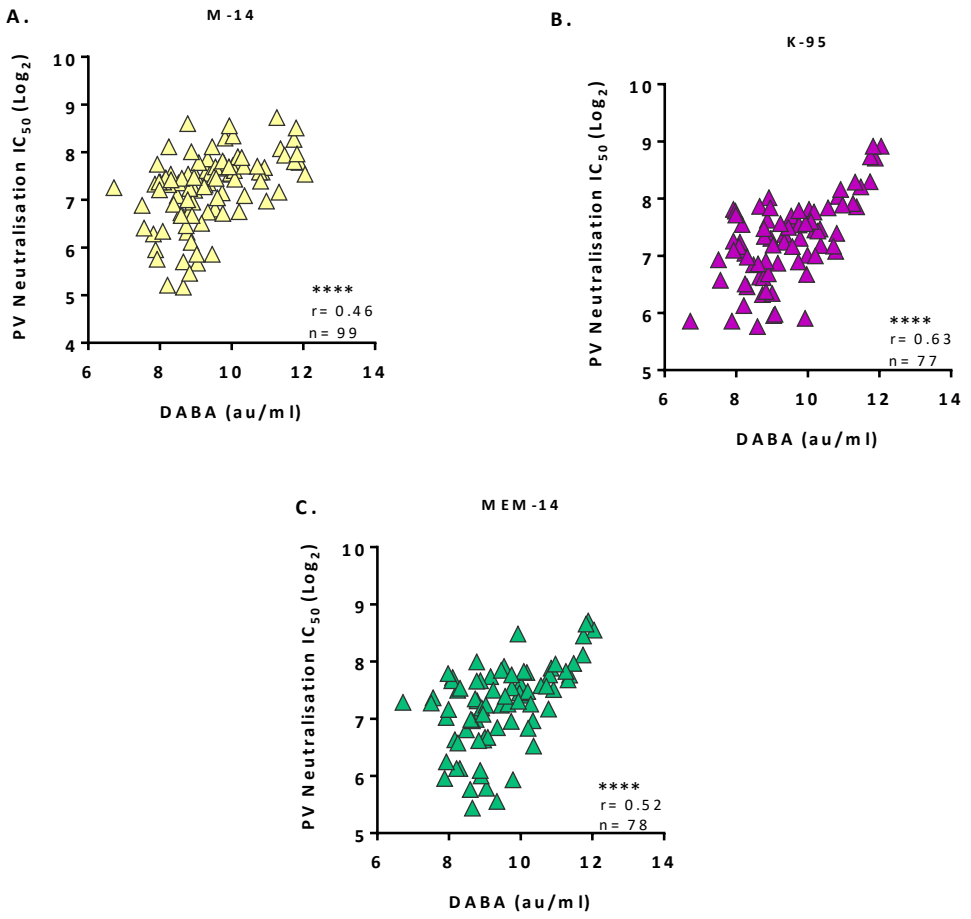


Figure 5.3 Correlation between EBOV PV neutralisation assay and DABA assay

Neutralisation IC_{50} titres measured with the EBOV PV neutralisation assay was observed to have a positive association A) M-14 r= 0.46, B) K-95 r= 0.63 and C) MEM-14 r= 0.52 with total anti-EBOV GP Abs present in CP, quantified by DABA. Pearson Correlation coefficients (r) was applied to the data after passing D'Agostino & Pearson omnibus normality test. A non-parametric Spearman Correlation was applied to A) M-14 as a normality test was not passed. The statistical significance of the correlation for all three EBOV strains was ****p< 0.0001.

Another point ascertained from the identified significant associations is that both the total Ab levels targeting EBOV GP; including IgM and IgG as well as the subset of NAbs within the total Ab population increase or decrease concurrently.

5.2.5 Neutralisation profile of IGH_EBOV CP Donor_045

As previously mentioned the donor samples included in the longitudinal analysis had neutralisation titres that could broadly be grouped into donors whose titres decreased or showed no distinctive trend. The majority of NAb trends demonstrated a decrease in titres however, one donor (IGH_EBOV CP Donor_045) showed a prominent increase with an overall remarkable neutralisation profile. The donor provided seven CP samples over a 154 day period; the first at 186 DPC and the last at 340 DPC. The first four samples were donated over a 54 day period; neutralisation potential of CP was observed to decrease between the first and the third sample, with the fourth sample having similar titres to the third. This decrease indicates NAb titres within these CP sample were losing their potency. However, the fifth CP sample donated only 23 days after the prior sample had a notable increase in EBOV neutralisation IC_{50} titre which was maintained for the following two samples donated. This increase was seen for all PV strains A) M-14, B) K-95 and C) MEM-14 (Figure 5.4), however, was much less pronounced with the M-14 EBOV strain (Figure 5.4 A). It is important to note that each CP sample was tested for EBOV RNA a minimum of two times by Public Health England (PHE) according to their regulations and were found to be EBOV RNA negative.

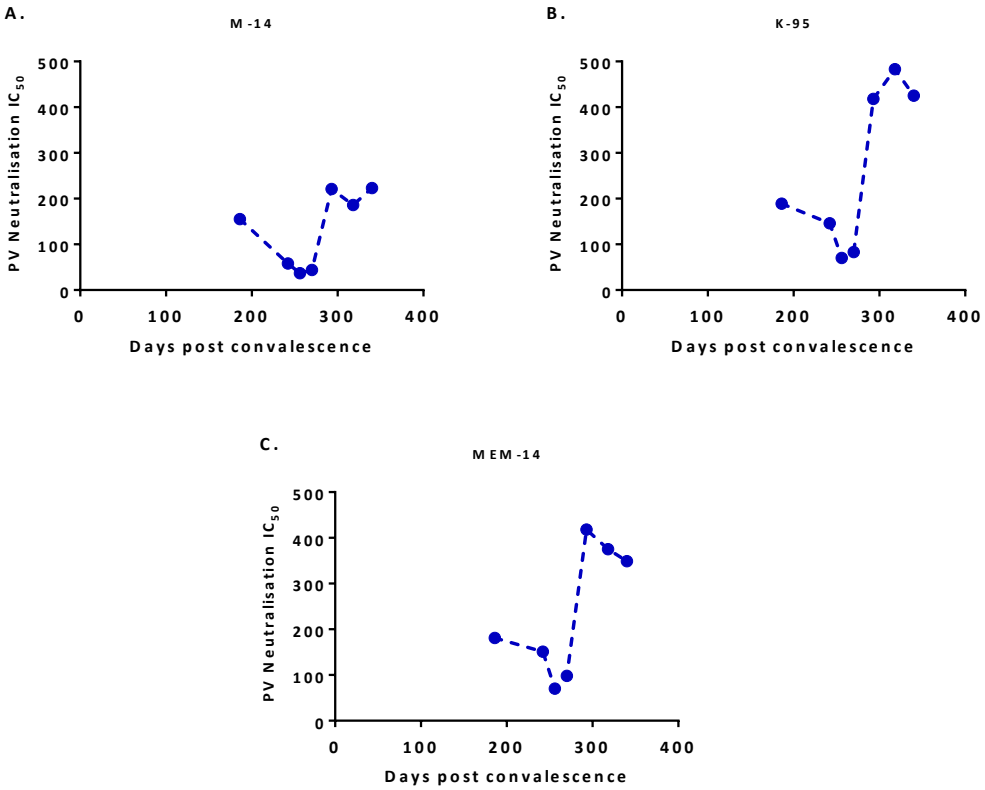


Figure 5.4 IGH_EBOV CP Donor_045: EBOV PV NAb profile

The seven CP samples from IGH_EBOV CP Donor_045 were measured with the three strains of the EBOV PV assay; A) M-14, B) K-95 and C) MEM-14 and were plotted against Days post convalescence (Day 0 standardised; donor tested double negative for EBOV).

5.2.6 RCE and total anti-EBOV Ab titre profile of IGH_EBOV CP Donor_045

To explore the cause of this striking neutralisation profile, the extent to which the same trend was observed for the PRNT as well as the quantified total anti-EBOV Ab titres present in the same CP samples were assessed. The PRNT neutralisation titres and the quantified Ab levels for each CP sample of IGH_EBOV CP Donor_045 were superimposed onto the graphs with EBOV PV neutralisation titres, respectively (Figure 5.5).

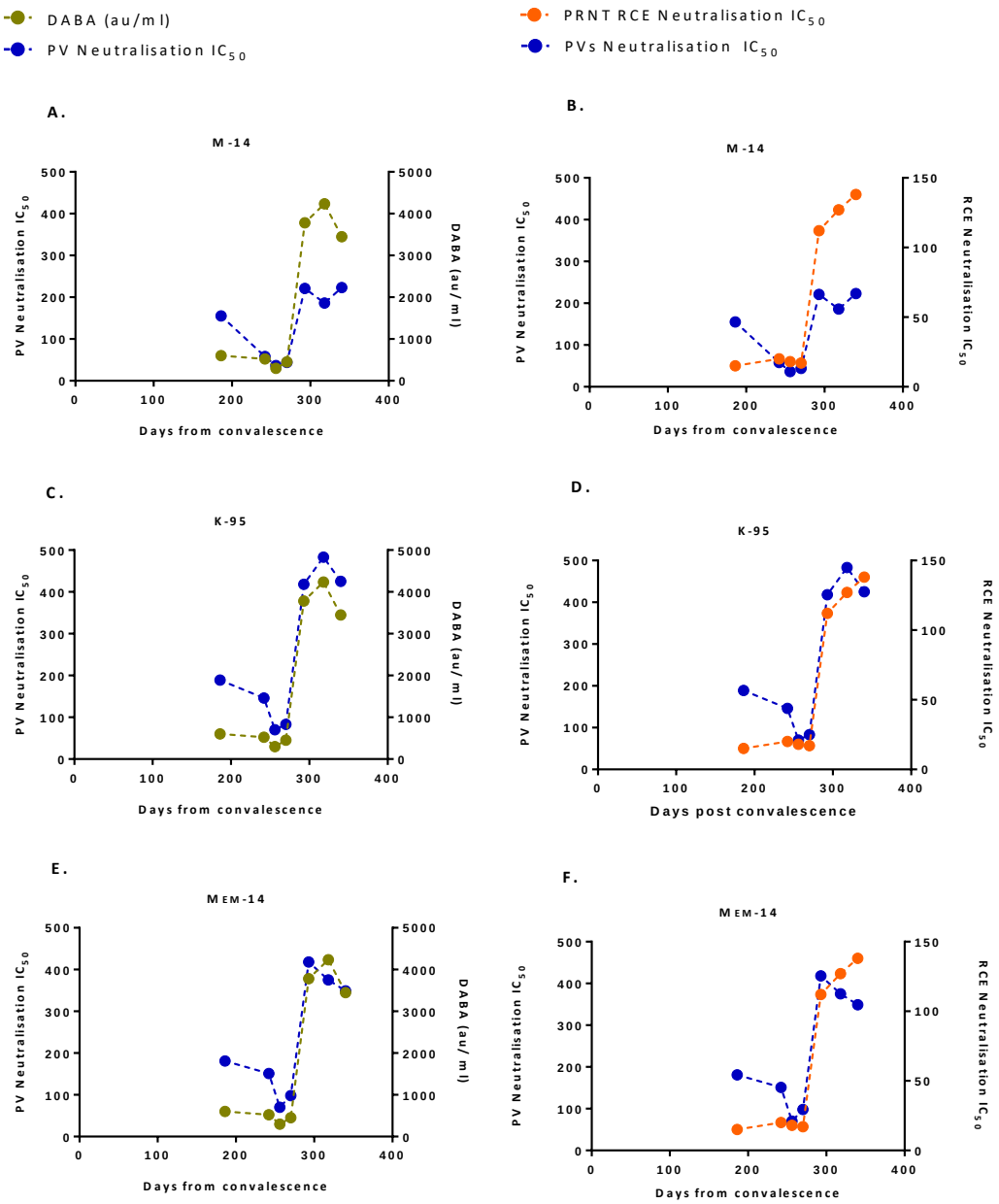


Figure 5.5 IGH_EBOV CP Donor_045: NAb and total Ab profile

The seven CP samples from IGH_EBOV CP Donor_045 were measured with DABA and RCE PRNT. The total quantified anti-EBOV Abs measured by DABA (green dots) A) M-14, C) K-95 and E) MEM-14 as well as the NAb titres measured with the RCE PRNT assay (orange dots) B) M-14, D) K-95 and F) MEM-14 were plotted for each respective sample alongside the NAb titres measured with the EBOV PV assay (blue dots).

EBOV GP targeting Abs quantified by DABA were observed to have a very close resemblance to the PV neutralising titres for EBOV strains C) K-95 and E) MEM-14 (Figure 5.5). Although not identical, the same trend could be observed for all three EBOV strains; the first four CP samples have lower Ab titres followed by an increase in the EBOV GP targeting Ab response of the donor. The results from the three individual assays which complemented each other suggested it was highly plausible that Donor_045 did indeed have a rapid surge in anti-EBOV Ab production over a 23 day period.

The above finding raised the question as to what could have induced this spike in Ab production. The three most conceivable notions were: the donor was re-infected with EBOV, the donor had an EBOV antigenic stimulation; which possibly resided in immune privileged sites to evade immune detection or lastly the donor took part in a vaccine trial without disclosing this information during CP donations for the study.

The last three plasma samples from Donor_45, which demonstrated potent neutralisation, also showed the highest quantities of total anti-EBOV Ab titres of the cohort. These, however, were observed not to be as effective at inhibiting the M-14 EBOV virus strain when compared to the K-95 and MEM-14 strains. Comparison of the CP samples with the lowest and highest quantified total anti-EBOV Ab titre for Donor_045, revealed that the higher titre had 14x more anti-EBOV activity than the lowest titre as measured by DABA. This vast increase in Ab titres over a short period as well as the differential inhibition of the M-14 EBOV PV, pointed towards the donor participating in a vaccine trial. There were two vaccine trials occurring around the same period in Sierra Leone; the Ad5-EBOV and the Ad26-ZEBOV+MVA-BN Filo trials (Wang et al., 2017). Hence the more potent CP Ab titres could be attributed to a boost in Abs as a result of an administered vaccine.

5.2.7 Follow up enquiry with longitudinal donors

Following on from this above rationale it was decided, to ask the donors through a questionnaire whether they had felt sick or whether they had participated in a vaccine trial. However, due to the period of time elapsed between CP donation and follow up (approximately 2 years) and the groundwork required in trying to pursue the donors to complete a full questionnaire, it was deemed feasible to gain responses only if questions were restricted. The two questions asked to donors who were attainable as well as their responses are summarised (Table 5.3).

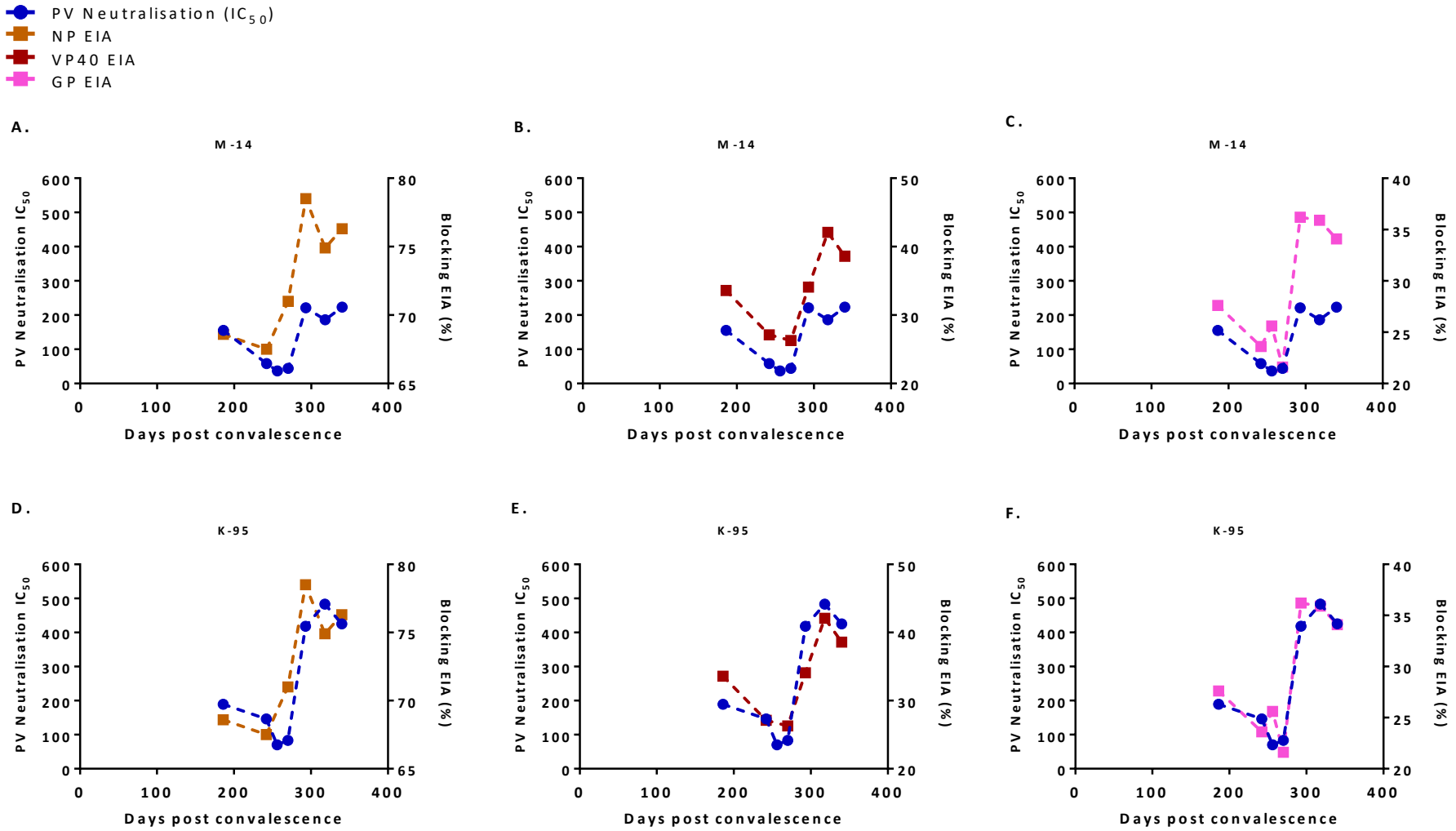
Table 5.3 Enquiry of vaccination study participation of longitudinal donors

Donor ID	Question 1	Question 2
	At the time when you were donating plasma for Ebola_CP (2015) were you given any vaccine?	At the time when you were donating plasma for Ebola_CP were you part of an Ebola vaccine study?
IGH_EBOV CP Donor_002	No	No
IGH_EBOV CP Donor_003	No	No
IGH_EBOV CP Donor_004	No	No
IGH_EBOV CP Donor_005	No	No
IGH_EBOV CP Donor_006	No	No
IGH_EBOV CP Donor_007	No	No
IGH_EBOV CP Donor_008	No	Yes Hepatitis Vaccine study
IGH_EBOV CP Donor_010	No	No
IGH_EBOV CP Donor_013	No	No
IGH_EBOV CP Donor_018	No	No
IGH_EBOV CP Donor_019	No	No
IGH_EBOV CP Donor_020	No	No
IGH_EBOV CP Donor_021	No	No
IGH_EBOV CP Donor_026	No	No
IGH_EBOV CP Donor_030	No	No
IGH_EBOV CP Donor_042	No	No
IGH_EBOV CP Donor_045	No	No
IGH_EBOV CP Donor_049	No	No

The follow up enquiry with the longitudinal donors summarised that responsive donors had not participated in any vaccine study. In addition, the co-ordinator of the Ebola-CP trial had received confirmation from the co-ordinators of the two Sierra Leone vaccine studies that were taking place at the same time stating that the donors were not eligible for inclusion in their vaccine studies. The steep increase in anti-EBOV Ab titres observed in Donor_045 is therefore more likely to be the result of an EBOV antigenic stimulation either from re-acquisition or re-emergence of EBOV.

5.2.8 ELISA detection for Abs against EBOV viral proteins NP and VP40

The next aim was to distinguish which possibility, re-infection from circulating EBOV or an antigenic stimulation of EBOV within the donor was more probable. In the first four CP samples, Donor_045 had shown circulating NAbs titres which would likely provide protection, in addition Sierra Leone had been declared 'Ebola free' on 15th November 2015 with the fourth sample of Donor_045 being donated early January 2016. This leaves the option of re-emergence of EBOV from an anatomical site where the virus may have persisted. To investigate this further, it was therefore decided to determine whether Ab titres increased against other EBOV proteins. EIAs were specifically developed to detect Abs to EBOV NP and VP40 in CP samples as well as an in-direct EIA to detect anti-EBOV IgG Abs (Jacobs et al., 2016) (Figure 5.6). NP is the main nucleocapsid protein of EBOV which directly encapsidates the viral genome (Takamatsu et al., 2018). The formation of the nucleocapsid occurs in inclusion bodies in the perinuclear region of EBOV infected cells, which act as virus factories (Takamatsu et al., 2018). VP40 is crucial for the recruitment of nucleocapsid to the cell periphery and for the budding of progeny virions (Takamatsu et al., 2018).


PV Neutralisation (IC50)
NP EIA
VP40 EIA
GP EIA
A.
M-14
B.
M-14
C.
M-14
D.
K-95
E.
K-95
F.
K-95
PV Neutralisation IC50
Blocking EIA (%)
Days post convalescence
0
100
200
300
400
600
500
400
300
200
100
80
75
70
65
50
40
30
20
35
25

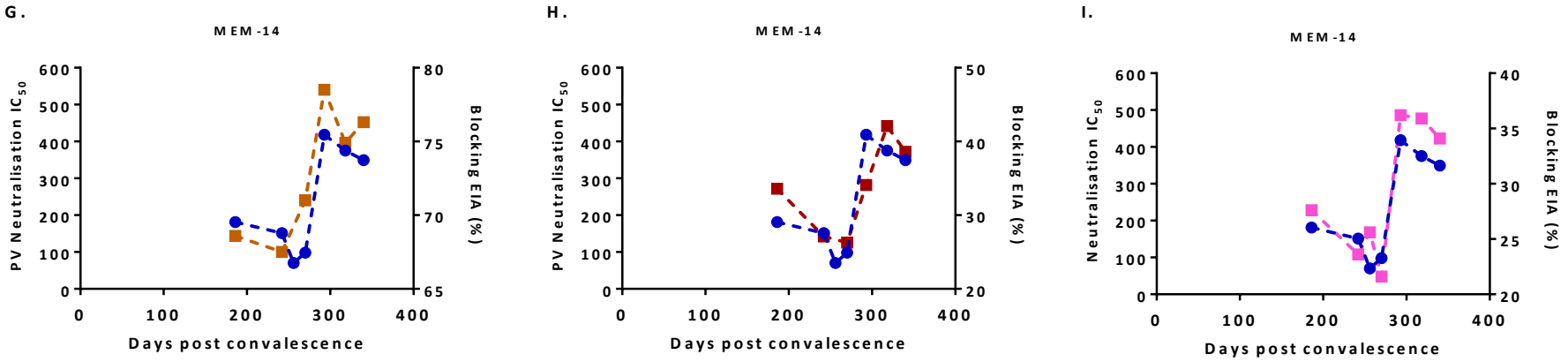
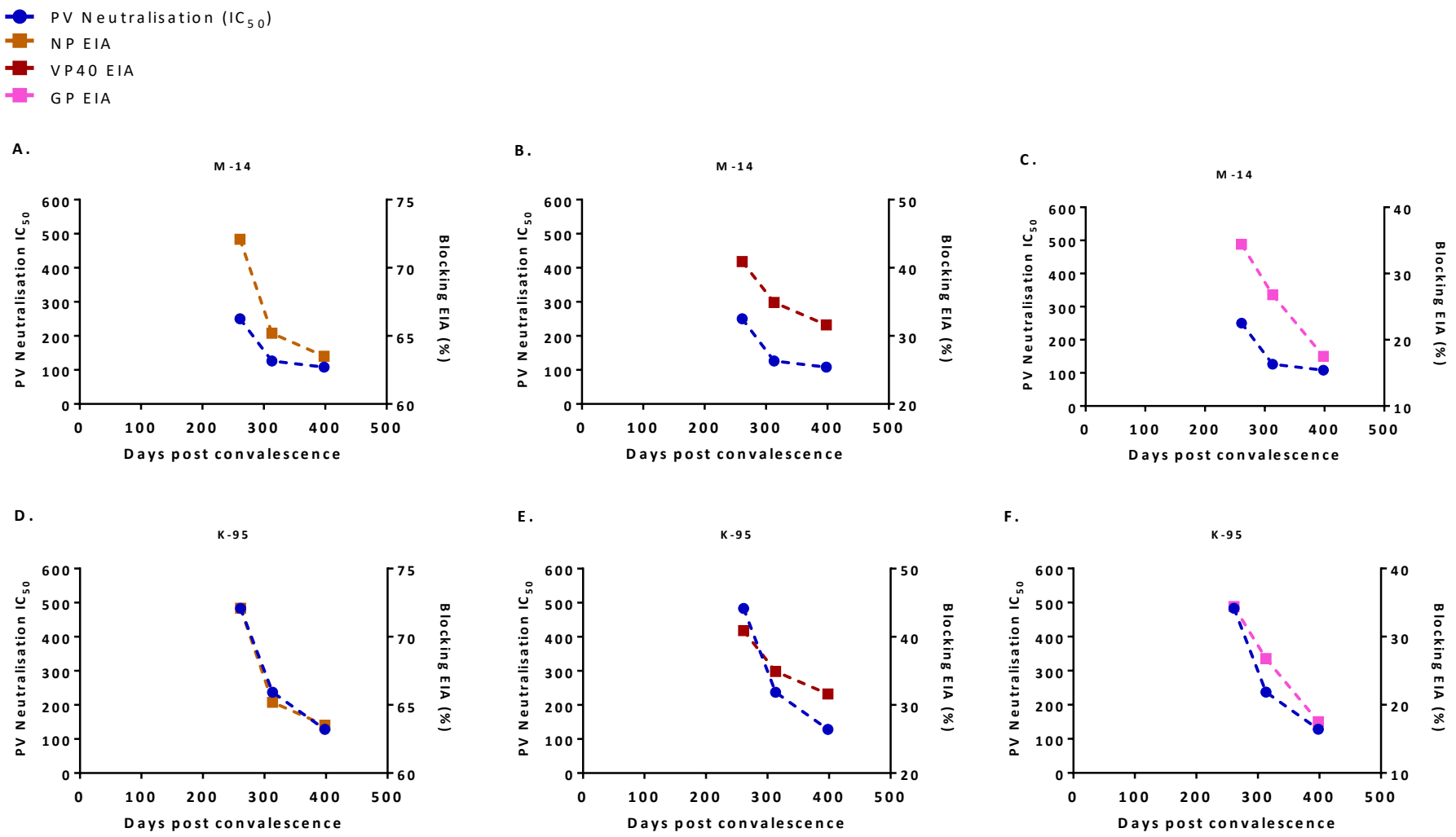


Figure 5.6 EIAs for the detection of EBOV NP, VP40 and IgG Abs to replication competent EBOV

Blocking EIAs using RCE were carried out for the detection of Abs to EBOV Nucleoprotein (NP) and viral matrix protein 40 (VP40). An indirect ELISA was carried out for the detection of anti-EBOV IgG. The results of these EIAs were plotted alongside the EBOV PV neutralisation IC_{50} titres for IGH_EBOV CP Donor_045 for each of the EBOV strains (blue dots). Anti-NP Ab results shown in A), D), G) (brown squares), anti-VP40 Ab results B), E), H) (red squares) and anti-IgG C), F), I) (pink squares).

The results of the EIAs revealed a similar trend for Donor_045 as seen previously with the PV and RCE PRNT, as well as the anti-EBOV Ab titres measured by DABA. Abs to EBOV NP, (Figure 5.6 A, D and G) and VP40 (Figure 5.6 B, E and H) were observed to decrease, increase and decrease again as previously observed. An EIA was carried out to determine total anti-EBOV IgG against RCE which also revealed the same NAb trend seen with NAb titres measured with the PV assay (Figure 5.6 C, F and I). The same trend was observed for all three EBOV strains; M-14, K-95 and MEM-14.

To assess if trends seen with the EIAs detecting Abs to NP, VP40 and GP were specific for Donor_45, a comparative experiment was conducted with CP samples from another donor within the cohort. Donor_49 was observed to have the opposite NAb trend to Donor_45; a prominent decrease in EBOV PV NAb titres with each subsequent CP sample donated. The EIA results also exhibited steep decreases in Abs to EBOV proteins NP (Figure 5.7 A, D and G), VP40 (Figure 5.7 B, E and H) and GP (Figure 5.7 C, F and I) over the period of the CP donation thus supporting that the results observed in Donor_45 were valid (Figure 5.4).


PV Neutralisation (IC50)
NP EIA
VP40 EIA
GP EIA
A.
M-14
PV Neutralisation IC50
Blocking EIA (%)
Days post convalescence
B.
M-14
PV Neutralisation IC50
Blocking EIA (%)
Days post convalescence
C.
M-14
PV Neutralisation IC50
Blocking EIA (%)
Days post convalescence
D.
K-95
PV Neutralisation IC50
Blocking EIA (%)
Days post convalescence
E.
K-95
PV Neutralisation IC50
Blocking EIA (%)
Days post convalescence
F.
K-95
PV Neutralisation IC50
Blocking EIA (%)
Days post convalescence

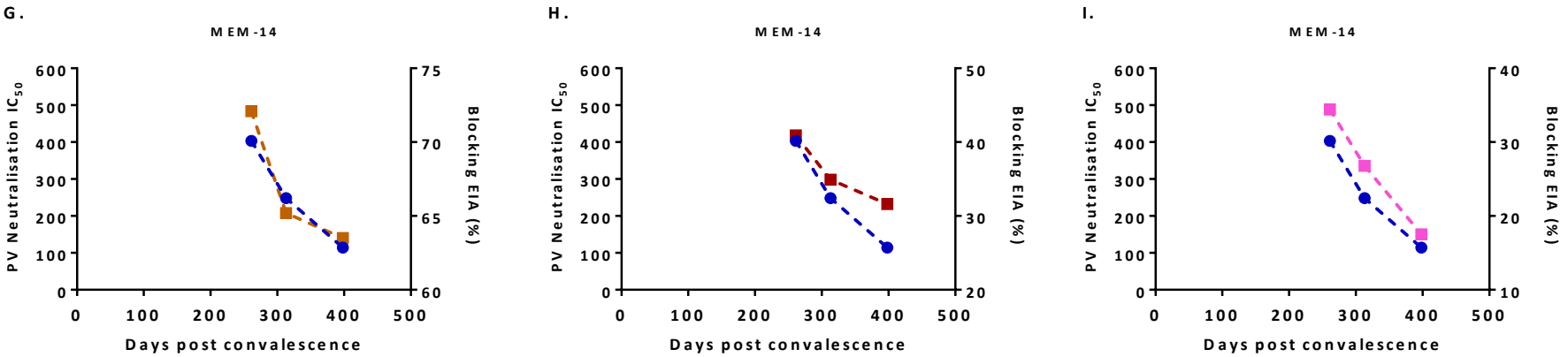


Figure 5.7 EIAs for the detection of EBOV Nucleoprotein, VP40 and IgG Abs to replication competent EBOV

Blocking EIAs using RCE were carried out for the detection of EBOV Nucleoprotein (NP) and viral matrix protein 40 (VP40). An indirect ELISA was carried out for the detection of anti-EBOV IgG. The results of these EIAs were plotted alongside the EBOV PV neutralisation IC_{50} titres for IGH_EBOV CP Donor_049 for each of the EBOV strains (blue dots). Anti-NP Ab results shown in A), D) and G) (brown squares), anti-VP40 Ab results B), E) and H) (red squares) and anti-IgG C), F) and I) (pink squares).

5.2.9 Doubling time and Half-life of CP NAbs

It was observed that some donors who provided longitudinal samples exhibited increases and decreases in NAb profiles (Figure 5.1). A mathematical analysis for calculating growth and decline was applied to the data to deduce inferences related to the time in which NAbs decreased. The total anti-EBOV Ab and NAbs of donors who provided three or more CP samples, previously measured with the PV neutralisation assay, RCE PRNT and DABA were utilised in this analysis. Both, growth and decline of NAb and total anti-EBOV Ab of EBOV survivors was inferred from the analysis, doubling time was found to be shorter than the half-life of NAbs for all assays, which was more prominent for the PV assay (Table 5.4) (Figure 5.8).

Table 5.4 Comparison of NAb doubling time and half-life

Median values (days) calculated for NAb doubling time and half-life for EBOV PV neutralisation assays with: M-14, K-95 and MEM-14, DABA and PRNT assay.

Parameter	Assays				
	M-14	K-95	MEM-14	DABA	PRNT
Doubling time (days)	30	28	14	77	63
Half-life (days)	92	99	87	87	82

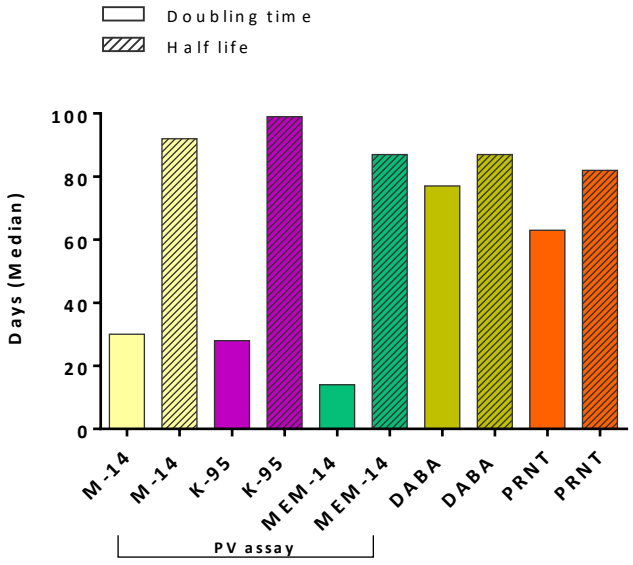


Figure 5.8 Doubling time and half-life of EBOV NAbs

Growth and decline functional analysis was applied to the CP EBOV NAb and total anti-EBOV Ab titres obtained with the PV neutralisation assay; M-14, K-95 and MEM-14, PRNT and DABA to calculate the doubling time (plain bar) and half-life (lined bar) in days of EBOV Abs in survivors.

5.3 Discussion

5.3.1 Longitudinal analysis of CP NAbs of Ebola survivors

EBOV specific Abs have been found to persist in EBOV survivors over a long period of time, with 40 years post-infection being the longest recorded (Rimoin et al., 2018). CP NAbs have also been observed to persist in our cohort; up to 505 days post convalescence (the latest time point sampled). It was previously concluded (4.2.5) that CP NAb titres differed between individual donors within the cohort, which was not attributed to the time at which the samples were donated. Assessment of the longitudinal CP samples utilising the EBOV PV neutralisation assay provided a better understanding of NAb titres in convalescent Ebola survivors. Diverse neutralisation CP profiles were observed, where donor NAbs could be broadly categorised into decreasing or no particular trend. However, the prominent differences in profiles within the same group indicated EBOV CP NAb titres was very much donor specific. The Ebola-Tx CP study had previously concluded that determining the levels of EBOV NAbs between donors was an important consideration for the effectiveness of CP EBOV intervention (van Griensven, Edwards, de Lamballerie, Semple, *et al.*, 2016). The longitudinal study described here indicates how greatly NAb titres can vary between donors and how the potency of titres can shift in a convalescent individual in a short period of time. Currently there is no literature regarding differential NAb potential between EBOV convalescent individuals. Although, research into HIV-1 infected individuals found that virus load, virus diversity and duration of infection independently influenced the development of bNAbs (Rusert et al., 2016). It has been previously documented that EBOV viral load equates to a higher mortality rate (Li et al., 2016), however, there has not been any documented associations between virus load and NAb titres. This would have been an interesting parameter to have investigated however, there was no access to patient clinical data.

5.3.2 Corroboration of three independent Assays

The three most widely employed assays for evaluating CP Ab titres are i) the PV neutralisation assay, ii) ELISA and iii) RCE PRNT, considered the 'gold standard'. The longitudinal study utilised all three assays for the evaluation and comparison of EBOV targeting Abs present in CP. Although the PV assay is commonly utilised due to the advantages previously discussed (1.10.3), one of the persistent criticisms of the assay is how representative it is of neutralisation when using replication competent virus. There is generally little opportunity for data acquired with the PV assay to be compared with a replication competent assay. The RCE PRNT was a blinded study; donor IDs of longitudinal CP samples used in this experiment

were coded, therefore, the researcher conducting the assay did not know which CP sample belonged to which donor in order to exclude researcher bias. This experimental setup adds further strength to the correlation observed between neutralisation titres measured with the PV assay and the replicative system. These results imply robust PV neutralisation assay which has been developed using HIV-1 structural proteins complemented with EBOV GP is measuring CP NAb titres is as effective as the 'gold' standard EBOV replicative assay.

The DABA EIA and PV neutralisation assay were also observed to positively correlate indicating an increase or decrease in total anti-EBOV Abs similarly resulted in an increase or decrease in NAb responses. The Ebola-Tx CP study assessed total anti-EBOV IgG and EBOV NAbs of 85 CP samples used in EVD patient CP transfusion and found that most CP contained anti-EBOV IgG (up to 1:25 000 titre), however, NAbs were low (5% had a titre of up to 1:60) in many donations (van Griensven et al., 2016a). The assays conducted in this study showed a wide range of neutralisation potential of NAbs with many donors showing potent titres (62.5% having a titre of 1:160 or higher) measured with the PV neutralisation assay. NAbs are known to be a proportion of a virus-specific Ab response, for example in flavivirus-infected individuals, <5% of total isolated Abs displayed potent (IC_{50} was able to be determined) neutralising activity (Vanblargan et al., 2016). It should, however, be noted that the DABA EIA assay utilised here measures all Ab subclasses (including IgM and IgG) and therefore is not specifically measuring IgG responses as measured in the other studies described. Since all plasma samples are taken sometime after viral recovery and IgM responses are known to be short lived (Ksiazek et al., 1999; McElroy et al., 2018) it is assumed that the responses measured using DABA are mainly IgG. It is poorly understood how Abs with different functional properties act together in human sera or plasma (Vanblargan et al., 2016), further investigation into this area may elucidate the Ab mechanisms that are vital in the immune response against EBOV.

5.3.3 A CP donor case study

The longitudinal analysis allowed us to analyse the individual CP NAb profiles of donors included in the study. Donor_45 contributed seven CP samples in total and had a unique neutralisation profile, which was observed to have a steep increase in Abs, which was confirmed between the PV, PRNT and DABA assays. The profile was attributed to participation in a vaccination study or antigenic stimulation of EBOV either from re-infection or from EBOV residing in an immune privileged site.

Participation in a vaccination study was ruled out as a possibility, leaving EBOV re-infection or antigenic stimulation as the two possible contributing reasons. The EIA results using replicative virus for Donor_45 and Donor_49 demonstrated the detection of Abs specific to NP, VP40 and GP. The 14 survivors of the 1976 Yambuku cohort who were tested for Abs to NP and VP40, were all found to have potent Abs to NP with only a few survivors retaining Abs to VP40 (Rimoin et al., 2018). Donor_45 and 49 were seen to have the same Ab trend to NP and VP40 as the PV NAb profiles. For Donor_49, as the NAb titres decreased so did Abs specific to NP and VP40 which showed there was a steep overall anti-EBOV Ab decrease in this donor over time. In contrast, Donor_45 was observed to have Abs to NP and VP40 which were similar to the NAb profile measured with the PV assay boosted between the 4^{th} and 5^{th} CP donation. NP plays a central role in viral replication and has been used as a target molecule for disease diagnosis (Wang *et al.*, 2012) whilst VP40 has a role in virus budding (Takamatsu et al., 2018). An increase in Abs specific to these EBOV structural proteins indicates an antigenic stimulation within the donor. As these CP samples also tested negative for EBOV RNA this indicates it was unlikely the donor had re-infection with EBOV from the external environment.

Following the 2013-2016 epidemic EBOV persistence has been documented in survivors, primarily when the individual exhibits sequelae and are EBOV positive. This was observed in the late severe relapse of a Scottish nurse who developed meningoencephalitis with cranial neuropathies and was found to have EBOV in cerebrospinal fluid (CSF) (Jacobs et al., 2016), EVD-related ocular complications is now widely recognised (Steptoe *et al.*, 2017), including cases where live EBOV has been detected within ocular tissues (Shantha et al., 2018) (Varkey et al., 2015). Cases have also arisen where survivors did not suffer post EVD-complications but harboured the virus in an anatomical site which subsequently led to transmission of the virus. One survivor had EBOV in seminal fluid 531 days post-symptom onset and was found to be the cause of a new cluster of EVD in Guinea and Liberia (Diallo et al., 2016), also an asymptomatic mother was able to transmit EBOV to her baby via breastmilk (Subissi, 2018). This case study has shown that although the CP donor tested EBOV RNA negative it is probable they were harbouring EBOV within a cryptic compartment where viral reactivation and antigen expression stimulated the immune system to heighten Ab responses against EBOV GP, NP and VP40 which may have curtailed the infection before any symptom onset. Viral proteins play an important role in host-virus interactions in EBOV infection (Rimoin et

al., 2018) therefore serological response to GP, NP and VP40 may be indicative of protection to EBOV. Furthermore it has been found that individuals who are NP and GP reactive appear more likely to neutralise EBOV in downstream applications (Rimoin et al., 2018) indicating Abs to these EBOV viral proteins may confer a protective benefit to the individual. The re-stimulation may also have been restricted to just antigen production and not the assembly of fully replicating competent virus. The documented cases of EBOV persistence highlights the potential impact that may arise if EBOV harbouring in immune privileged sites was activated and released into the periphery.

It is interesting that when comparing the heightened Ab responses of Donor_045 there appears to be differences between the EBOV strains, with M-14 shown to be weaker than for both the K-95 and MEM-14 strain. This could be attributed to the fact that the EBOV GP may have undergone amino acid alterations that have restricted the strength of boosting of Abs reactive against M-14. There are 4 amino acid differences between the M-14 and MEM-14 strain (Fig 2.1) which could therefore have contributed to the weaker induction.

5.3.4 Doubling time and Half-life life of NAb

The increased/ decreased NAb profile observed in some survivors who donated multiple CP samples, points towards a scenario where Ebola survivors are potentially exposed intermittently to EBOV antigens but are able to respond with adequate NAb titres, thereby preventing uninhibited EBOV infection. The mathematical analysis to calculate growth and decline of these increasing/ decreasing Ab profiles revealed a quicker Ab titre doubling time suggesting the immune system is able to boost Ab responses according to EBOV antigenic stimulations and that this immune response is sustained for a longer period as indicated by the slower rate of decline observed with the different assays. After primary exposure to an antigen, secondary antigen specific response rates are much quicker upon re-exposure (Microbe online, 2018); this could explain the prominent difference between growth rate and decline observed for the PV assay as only EBOV specific NAb titres are measured. However, it may take a longer period for other Ab produced in an immune response against EBOV to increase as was observed with the DABA assay. It would be expected that the PRNT measuring NAbs would show the same difference in growth rate as the PV assay, the same trend was observed; a faster growth/ decline Ab rate, however, the slower growth rate compared to the PV assay could potentially be attributed to a smaller data set.

5.3.5 Chapter conclusion

The work presented in this chapter showed that the longitudinal analysis of the Ebola survivor CP displayed diverse donor specific NAb profiles. Further analysis of increasing/ decreasing NAb profiles as well as the detection of Ab to EBOV proteins in Donor_45, revealed that donors may be subjected to periodic EBOV antigenic stimulation. This is possibly from virus residing in cryptic anatomical sites whereby, the quick production and sufficient levels of NAbs, controls infection which was supported by the Ab growth/ decline analysis. The similarity in neutralisation of the 2013 EBOV, 1995 Kikwit and the mutant strains point towards Abs produced in response to a specific EBOV strain being able to cross-react and neutralise EBOV variants.

6 EBOV soluble glycoprotein interaction with neutralising Abs of Ebola survivor convalescent plasma

6.1 Introduction

The EBOV Soluble Glycoprotein (sGP) is the primary product produced from transcription of the GP gene in approximately 70% of transcripts (de La Vega, Wong, Kobinger, *et al.*, 2015) and has been detected in abundance in the blood of EBOV infected individuals (Ksiazek et al., 1999). However, to date very little progress has been made in understanding the biological significance of sGP in relation to EBOV pathogenesis and consequently the impact it may have in EBOV treatment in infected individuals. The proposed role of sGP in EBOV pathogenesis was previously discussed in detail (1.9.2.1) with a summary shown below (Table 6.1).

Table 6.1 Summary of sGP roles in EBOV pathogenesis

Potential sGP Role	Description
Decoy antigen	sGP can bind to EBOV-specific immune responses thereby leaving EBOV free to infect target cells (Ito, Watanabe and Takada, 2001).
Antigenic subversion	B cells recognising epitopes shared between both the sGP and $GP_{1,2}$ isoforms, would more likely encounter sGP due to its abundance in EBOV infected individuals and therefore the immune response would be re-directed towards sGP, which would adsorb the Abs (Mohan *et al.*, 2012).
Lymphocyte apoptosis	sGP could cause bystander apoptosis of uninfected B and T cells which have not been targeted for EBOV infection (Wauquier *et al.*, 2010).
Neutrophil Inactivation	sGP binding and inactivation of neutrophils (Yang *et al.*, 1998).
Vascular dysregulation/ restoration of barrier function	Research has speculated that sGP could be responsible for endothelial cell changes and vascular dysregulation whilst conflicting research has indicated sGP may rescue barrier function activity (Wahl-jensen *et al.*, 2005).
Δ-peptide receptor blocking	Δ-peptide the cleavage product of sGP has been documented to inhibit EBOV and MARV virus entry into target cells (Miller *et al.*, 2012).

6.1.1 The impact of sGP as a decoy antigen

The work in this chapter focuses on understanding the role of sGP as a potential decoy antigen; which has the ability to bind to EBOV-specific immune responses. It has been documented that sGP can cross-react with Abs present in the sera of EBOV survivors (Ito et al., 2001) suggesting the necessity to investigate the role of sGP functioning as a decoy antigen and with relevance to EBOV treatment. The interaction of sGP with CP NAbs or Abs elicited from vaccines could potentially have detrimental effects on the overall immunity of the individual if re-/infected with EBOV or those who experience virus re-activation. The virulent nature of EBOV means prophylactic treatment of EBOV is a well sought after aim, however, if sGP has the potential to absorb NAbs then EBOV-specific Abs would be rendered less efficient and EBOV would be unrestricted to infect target cells.

6.1.2 Aims of this Chapter

The development of a well characterised EBOV GP PV neutralisation assay utilised for the evaluation of CP NAbs served as a good tool with which to mimic certain roles of sGP during natural EBOV infection. The PV system was used to assess the interaction of sGP with PV and in the presence of CP. A series of experiments were conducted in order to explore the following:

1. The effect of sGP effect on PV infectivity and production during co-production
2. CP neutralisation of co-produced sGP and EBOV PV
3. Effect of sGP on EBOV PV inhibition
4. Effect of sGP on EBOV PV CP neutralisation

6.2 Results

6.2.1 sGP co-production

6.2.1.1 sGP and EBOV GP dual production

Transcription of the EBOV GP gene primarily yields the non-structural sGP which is secreted from EBOV infected cells, however, a slippage of the L-polymerase during transcription results in the formation of the structural EBOV GP utilised in viral entry (Lee and Saphire, 2009). Both EBOV proteins can be produced within the same cell therefore the impact of sGP production on EBOV infectious titres was assessed *in vitro*. The standard 285 ng of M-14 plasmid (previously discussed in 3.2.3.6) was co-transfected alongside varying amounts (25-2000 ng) of the sGP plasmid. The resulting virus produced was harvested 72 h post-transfection and frozen at -80°C for 24 h prior to infection of TZM-bl cells. The sGP co-transfected virus was compared to M-14 virus infection without sGP (Figure 6.1).

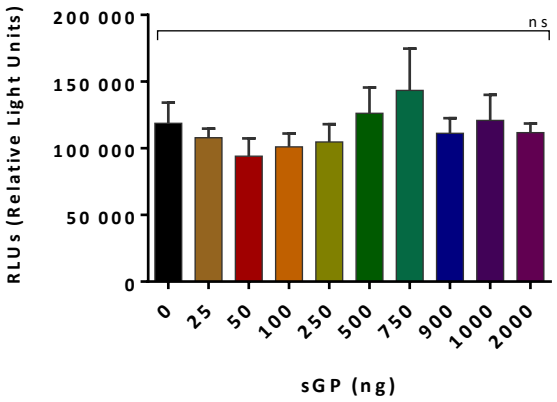


Figure 6.1 Co-transfection of EBOV sGP and M-14 GP

EBOV sGP plasmid was titrated (0-2000 ng) alongside 2000 ng of pSG3$^{\Delta Env}$ backbone and 285 ng of EBOV strain M-14 Env during the transfection process. The resulting virus stocks were frozen at -80°C prior to infection into TZM-bl cells and luciferase activity was measured 48 h post-infection. Non-parametric Kruskal-Wallis test revealed no significant differences in infectivity between the virus stocks. The experiment was repeated technically (n= 3) and biologically (n= 2), error bars indicate mean with standard deviation.

The infectivity of the sGP co-produced virus were analysed using a non-parametric Kruskal-Wallis test revealing that infectious titres were not significantly different from each other, indicating sGP did not have an effect on EBOV GP infectivity (Figure 6.1). Previously it was shown, p24, a HIV-1 capsid protein did not vary significantly between different batches of

EBOV PV which is indicative of the number of virus particles produced. A p24 ELISA was conducted on the co-produced virus which measured whether sGP affected EBOV PV production numbers. No statistical correlation was found when comparing p24 input of GP/ sGP co-produced PV with infectivity of TZM-bl cells (Figure 6.2).

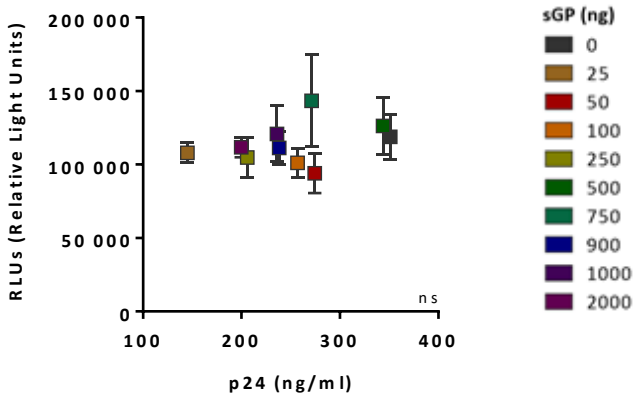


Figure 6.2 Correlation of p24 quantitation with corresponding infectivity of sGP co-transfected virus

EBOV M-14 PV co-produced with titrated sGP (0-2000 ng) and pSG3$^{\Delta Env}$ (2000 ng) was quantified by p24 capsid ELISA (colour corresponds to sGP input shown in fig 6.1). The p24 concentration (technical repeat n= 3) was assessed against corresponding infectivity titres with a correlation analysis, no significant differences were observed. Error bars indicate mean with standard deviation.

Three of the sGP co-transfected stocks; 25, 250 and 900 ng sGP input were selected for use in a neutralisation assay alongside M-14 produced virus without sGP. IGH_EBOV CP Donor_042 was utilised as it was previously observed to be a potent EBOV neutraliser. EBOV PV co-transfected with sGP was not observed to have an effect on EBOV infectivity nor the number of virus particles produced. The production of sGP would result in the EBOV protein being secreted along with the PV from transfected cells. Therefore, the neutralisation of the PV/ sGP supernatant was assessed for neutralisation with EBOV survivor CP. The plasma was serially diluted and incubated with the PV stocks containing sGP prior to infection of TZM-bl cells and where luciferase activity was measured 48 h post-infection. Neutralisation of sGP co-transfected virus with Donor_042 CP was compared to M-14 neutralisation alone as well as neutralisation with EBOV negative plasma.

The neutralisation curves show that all virus stocks incubated with the EBOV negative plasma did not inhibit virus entry (Figure 6.3 A). The CP neutralisation of M-14 virus stocks without sGP can be seen to have an overall greater inhibitory profile in comparison to neutralisation of the three M-14 stocks with sGP present. To visualise better the differences, IC_{50} titres were extracted from the neutralisation curves and plotted as a bar graph (Figure 6.3 B). M-14 (yellow bar) labelled as 0 ng sGP had the highest IC_{50} titre inferring less Ab was required to inhibit 50% of EBOV PV compared to the other EBOV PV/ sGP stocks. A trend was observed; IC_{50} titres decreased as sGP input in transfection increased (brown, green, teal bars respectively) (Figure 6.3 B) thus indicating more Ab was required for 50% inhibition of the virus stocks with increasing concentrations of sGP likely present. Statistical analysis was unable to be performed due to a small data set however the experiments was performed twice and the same results were observed.

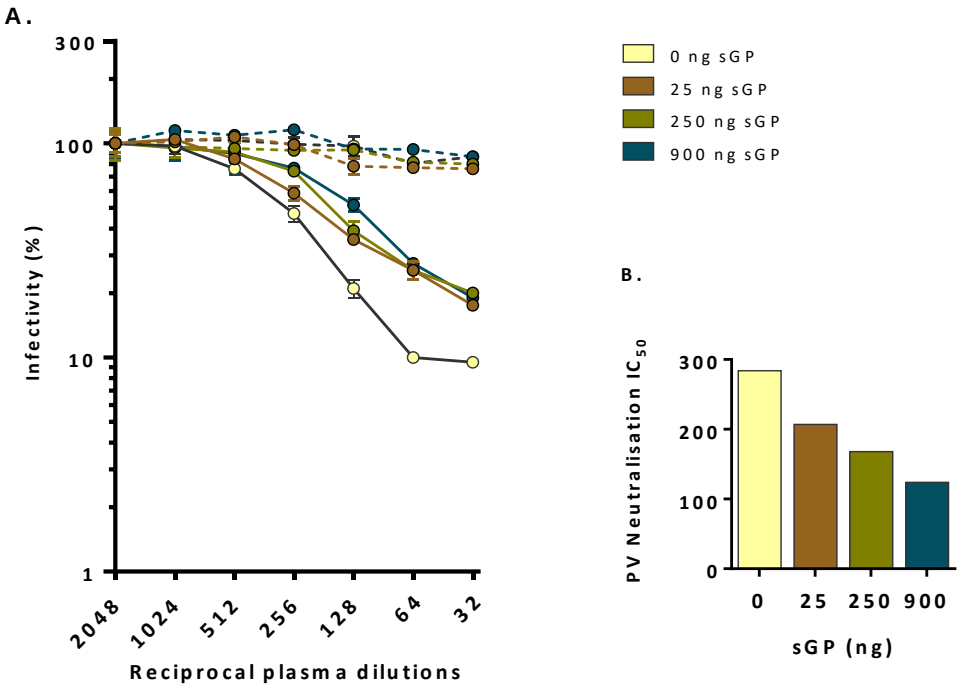


Figure 6.3 CP neutralisation of sGP co-transfected PV

A) M-14 PV alone (yellow) and M-14 PV co-produced with 25 (brown), 250 (green), 900 (turquoise) ng of sGP was utilised in a neutralisation assay with Donor_042 CP (straight line) as well as EBOV negative plasma (dotted line). The experiment was repeated technically (n= 2) and biologically (n= 2) and error bars indicate mean with standard deviation. B) IC_{50} titres extracted from the neutralisation of M-14 PV alone and M-14 PV co-produced with sGP (25-900 ng) (graph A) were plotted as a bar graph to illustrate the difference in CP neutralisation of the virus stocks.

6.2.2 In-house sGP production

The sGP expressed during EBOV PV co-production was deemed to be functional since differential neutralisation with CP was observed. Characterisation of sGP, including quantification of the protein, would require separation from EBOV PV, however, this was deemed problematic given that no sGP specific Abs were available.

6.2.2.1 Optimisation of sGP production

sGP was first produced in DMEM complete media, where supernatant was harvested 72 h post-transfection, aliquoted and frozen. FE25 (ENZO), an Ab targeting the EBOV GP, was used for the detection of sGP alongside a positive and negative control, EBOV PV and cell supernatant, respectively. The Immunoblot revealed non-specific binding of the Ab to an abundant protein present in all three samples; most likely FBS (result not shown).

Following this, sGP was produced in DMEM containing no FBS, 50% FBS and specialised serum free media (SFM) (Gibco) which resulted in the quick detachment of cells. The best approach to maintain cell viability was to seed and replace the transfection mix with DMEM complete media following transfection then 12-15 h later again replace the media with FBS free DMEM or SFM (GIBCO) and harvest the protein 48 h later. M-14 transfected concurrently under the same conditions as sGP was used as a control to check the effect on virus infectivity (Figure 6.4). This method yielded infectious EBOV PV in FBS free DMEM and SFM, albeit at 50% reduced levels, possibly due to less glycosylation of the PV or decreased numbers of PV being produced. This result indicated that the transfection process was functional.

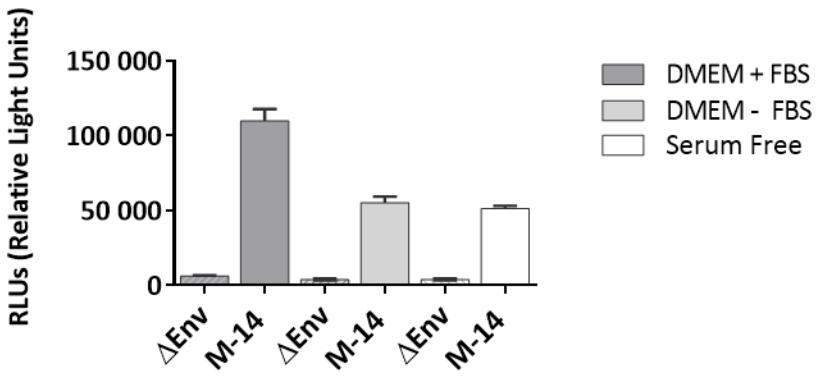


Figure 6.4 Production of EBOV M-14 PV in different media

EBOV PV strain; M-14 was produced in DMEM +/- FBS and Serum Free Media (GIBCO) and was subsequently frozen at -80°C prior to infection into TZM-bl cells. The effect of different media on PV production and infection was assessed. The experiment was reproduced biologically (n= 2) and technically (n= 4), error bars indicate mean with standard deviation.

A large batch of sGP produced either with FBS free DMEM or SFM were pooled and concentrated using the Amicon®Ultra-15 centrifugal filter devices (Merck Millipore, UK) with a 30,000 Molecular Weight Cut off (MWCO). The concentrate was exchanged into PBS, and subsequently quantified using the Bradford assay and an SDS-PAGE was run for subsequent immunoblotting.

6.2.2.2 Immunoblot of EBOV sGP

Two batches of concentrated M-14 PV and sGP were run using SDS-PAGE, and the membranes probed with IGH_EBOV CP Donor_045 plasma (high neutralising CP activity) as commercial anti-EBOV Abs (FE37, FE25 (ENZO)) and FE18 (ThermoFisher) were observed to non-specifically bind in immunoblots (see appendix). Supernatant of cells alone which went through the same concentrating process as sGP was used as a negative control, labelled Neg (Figure 6.5). PV and sGP were produced in both A) FBS free DMEM and in B) SFM (Figure 6.5). No bands were detected in the negative control, however, a stronger band at 150 kDa was detected in the M-14 PV produced in SFM (especially batch 1) compared to PV produced in FBS free DMEM. The sGP was detected at approximately 102 kDa in both media preparations (Figure 6.5).

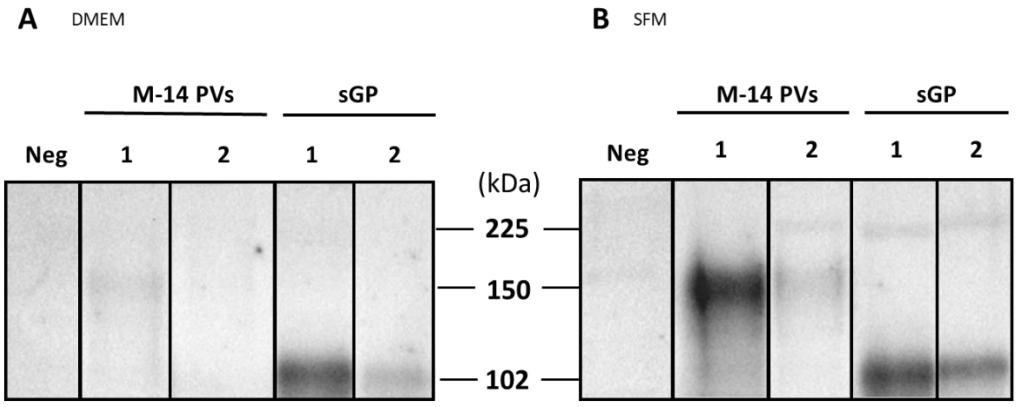


Figure 6.5 Immunoblot for the detection of sGP and M-14 PV

Two batches of M-14 PV, sGP and the negative control; supernatant of cells that underwent the transfection process without any plasmids were produced in A) DMEM (–FBS) and B) SFM (GIBCO). The membranes comprising the proteins (A + B) were probed with CP of Donor_045. Bands for sGP were detected at 102 kDa for both membranes. No band was detected for M-14 PV produced in DMEM and a band of 150 kDa was detected for batch 1 of M-14 PV produced in SFM. No bands were detected in either of the negative controls.

6.2.2.3 Inhibition of EBOV PV with EBOV sGP

Following confirmation of sGP production, its ability to inhibit EBOV PV entry was tested. Serial dilutions of sGP were incubated with M-14 PV prior to infection of TZM-bl cells. The positive and negative controls; PV incubated with IGH_EBOV CP Donor_018 and negative human plasma respectively, were run concurrently (Figure 6.6 A) with M-14 PV incubated with B) sGP produced in DMEM, 108-2 µg and C) SFM, 232-4 µg. The positive control inhibited M-14 whilst the negative control did not, as expected (Figure 6.6 A), however, the sGP produced in both DMEM and SFM also did not inhibit PV infectivity (Figure 6.6 B + C).

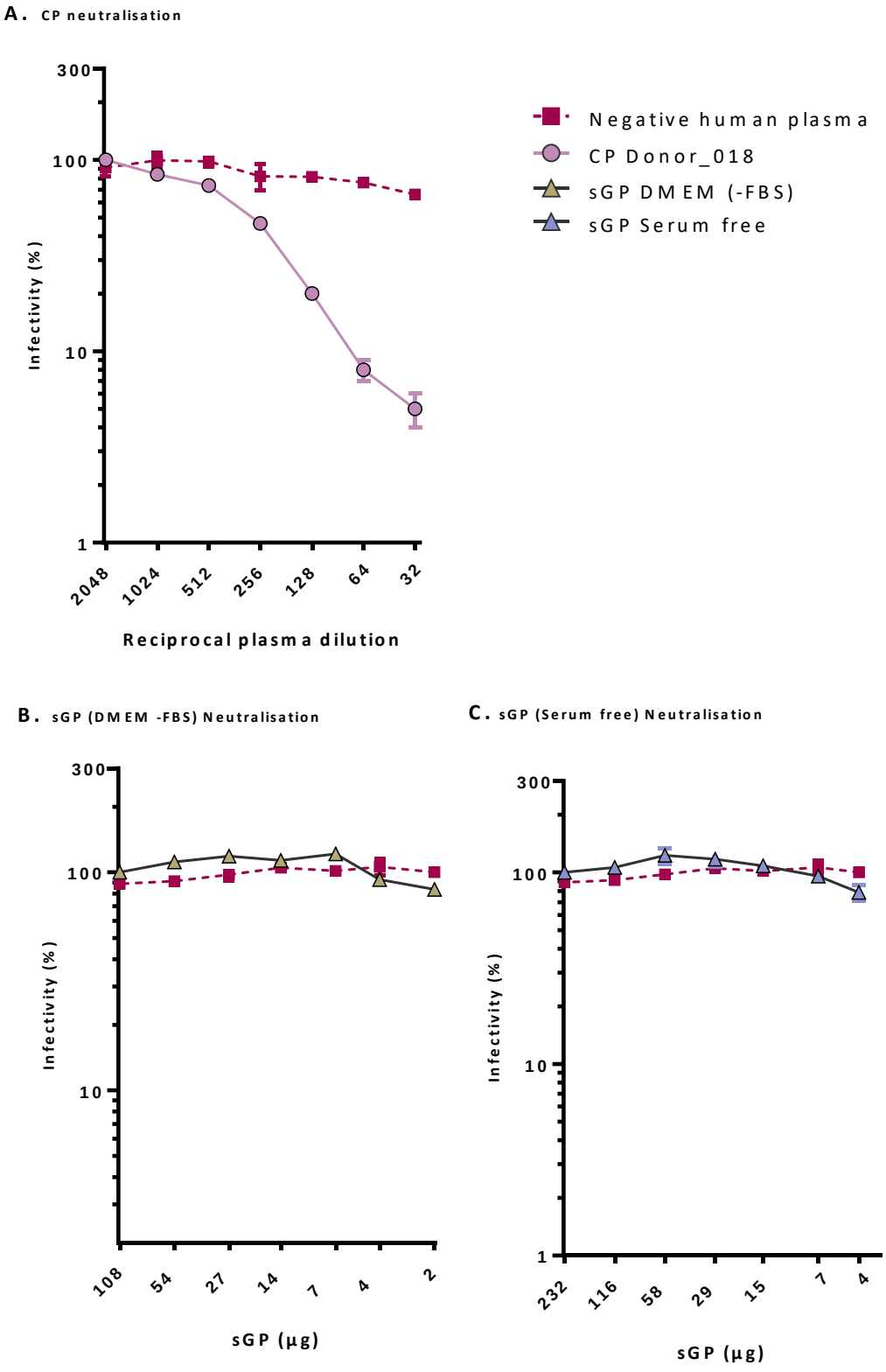


Figure 6.6 Neutralisation of EBOV PV with EBOV sGP

Neutralisation of EBOV strain M-14 PV with A) CP Donor_018, B) sGP produced in FBS free DMEM (108-1.69 µg), C) sGP produced in serum free media (GIBCO) (232-4 µg) and EBOV negative human plasma (pink squares). Biological repeats were conducted (n= 3) and technical repeats (n= 2), error bars represent mean with standard deviation.

6.2.2.4 Inhibition of EBOV PV with EBOV CP in the presence of sGP

It was previously observed that CP could differentially neutralise sGP co-transfected virus dependent on the input of sGP. This experiment was repeated using the concentrated sGP produced in FBS free DMEM and SFM using both high (Donor_018) and low (Donor_047) neutralising CP samples, with sGP serial dilutions ranging from 108-2µg for DMEM produced sGP and 232-4µg for SFM sGP. The experiment was undertaken to see if any inferences could be made between neutralisation of EBOV PV with different CP potency in the presence of sGP. It was observed that the high neutraliser CP (Donor_018) showed slightly less overall neutralisation in the presence of both types of sGP produced (Figure 6.7 A) which was reflected in the bar chart depicting neutralisation IC_{50} titres. However, the lower CP neutraliser (Donor_047) did not show a difference between neutralisation of M-14 PV with sGP (Figure 6.7 B). Although high quantities of sGP were tested in the neutralisation assays it did not affect PV infectivity as previously observed (Figure 6.6) thus sGP may not be concentrated enough to measure differences. Statistical analysis was unable to be performed due to a small data set however, the experiment was performed twice and the same trends were observed.

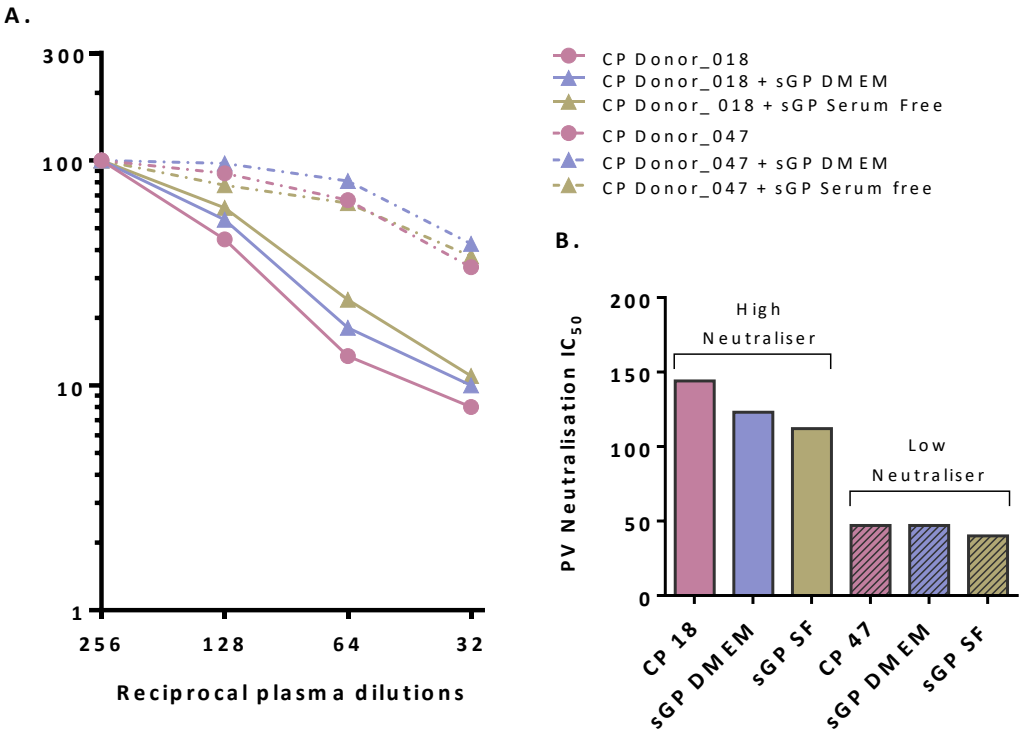


Figure 6.7 Inhibition of EBOV PV with EBOV sGP in the presence of CP

A) Neutralisation of M-14 PV with CP (pink); high neutralising CP, Donor_018 (lines) or low neutralising CP Donor_047 (dotted lines) in the presence of sGP produced in FBS free DMEM (purple) or SFM sGP (brown). Biological repeats were conducted (n= 1) and technical repeats (n= 2). B) IC_{50} titres extracted from the neutralisation of M-14 PV with CP alone (CP 18 and CP 47- pink) and CP in the presence of sGP (DMEM-purple and SFM-brown produced) were plotted as a bar graph to illustrate the difference of M-14 PV neutralisation in the different conditions.

6.2.3 Commercially produced sGP

The experiments conducted utilising in-house generated sGP suggested co-production of GP and sGP did not affect PV infectivity or CP neutralisation, however, due to the problematic nature of detecting co-produced sGP and the possible loss of sGP function during production these results were deemed non-conclusive. To further investigate the effects of sGP on EBOV infectivity and neutralisation, experiments were conducted using commercially produced EBOV sGP. Due to availability at the time of the experiment subtype Zaire strain Kikwit 1995 (2BScientific) was utilised in the following assays.

6.2.3.1 Immunoblot of commercially produced sGP

The commercially produced sGP protein was tested using SDS-PAGE and Immunoblot analysis. Supernatant from cells transfected alone was run on an SDS-PAGE alongside 1 µg, 0.5 µg, 0.1 µg and 0.05 µg (lanes 1-4 respectively) of commercially produced sGP and subsequently probed with CP (Donor_045). Bands were detected at 150 and 17 kDa in lanes 1 and 2; 1 and 0.5 µg sGP, respectively, no bands were detected in the negative control (Figure 6.8).

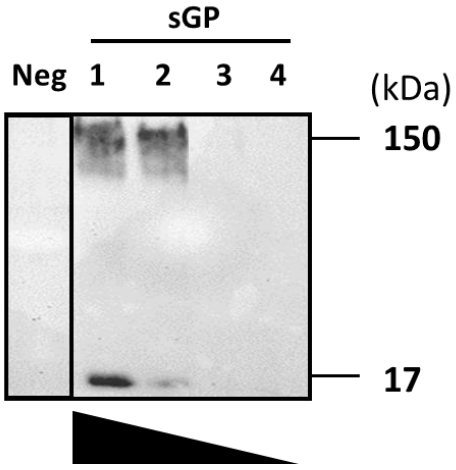


Figure 6.8 Immunoblot of commercially produced sGP

Immunoblot detection of commercially produced sGP 1, 0.5, 0.1, 0.05 µg in lanes 1-4 respectively with CP Donor_045. Two bands were detected in lanes 1 and 2 each at 150 kDa and 17 kDa. A negative control supernatant of transfected cells alone was probed alongside sGP, no band was detected.

6.2.3.2 Controls implemented for commercially produced sGP experiments

Due to the complexity of the PV CP neutralisation assays in the presence of sGP, extensive controls were implemented to ensure the data acquired was the result of sGP function in the individual experiment. PV was incubated with the respective sGP concentrations utilised in the experiment; 0.03, 0.16 and 0.33 µg/ well was used in this experiment prior to infection into TZM-bl cells (Figure 6.9 A + B). A Kruskal- Wallis test used to scrutinise the data revealed there were no significant differences between PV incubated with the differing concentrations of sGP (Figure 6.9 A + B (control 1)). EBOV negative plasma was incubated with PV at the same CP IC_{50} dilution used for each individual experiment prior to infection into TZM-bl cells. The luciferase activity of PV incubated with negative plasma alone was compared to PV incubated with the respective negative plasma dilution and sGP. A non-parametric, Mann-Whitney U test revealed no significant differences between the controls (Figure 6.9 A + B (control 2 +3)). These controls indicate that sGP alone did not have an effect on PV infectivity nor did sGP interact with EBOV negative plasma affecting infectivity. These sets of controls are representative for all following experiments conducted with sGP and CP.

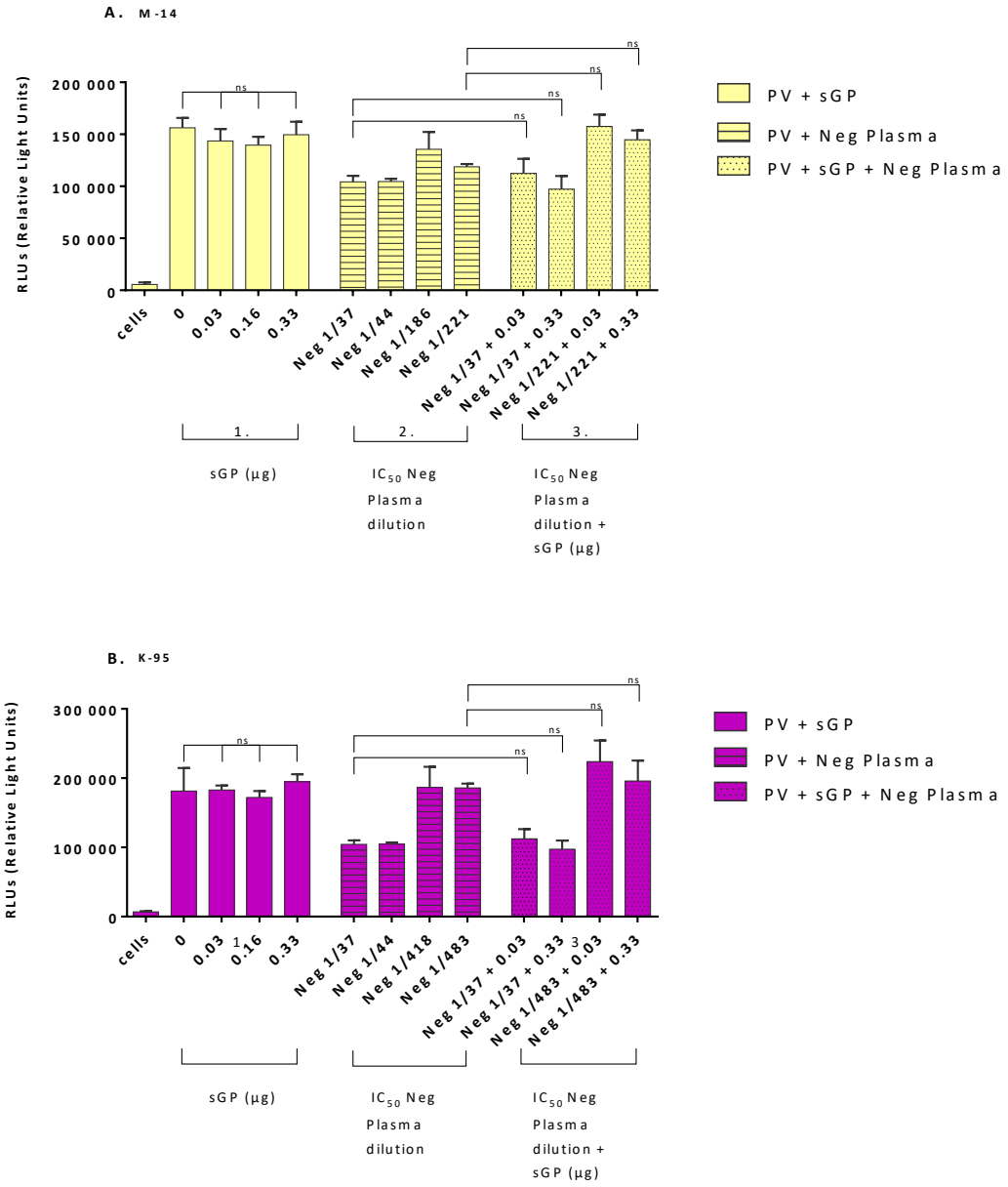


Figure 6.9 Set of controls implemented for sGP experiments

Extensive controls were applied to individual sGP experiments. 1) PV was incubated with the respective sGP concentrations prior to infection into TZM-bl cells. A Kruskal-Wallis test revealed no significant differences in PV incubation with differing concentrations of sGP. 2) Negative plasma at the same CP IC_{50} dilution used in the experiment was incubated with PV prior to infection into TZM-bl cells. 3) The respective negative plasma dilution and sGP was incubated with PV prior to infection into TZM-bl cells. A Mann-Whitney U test revealed no significant differences between PV incubated with negative plasma alone and negative plasma with sGP. These controls were conducted for both A) M-14 and B) K-95 EBOV strains. These sets of controls are representative for all experiments conducted with sGP and CP.

6.2.3.3 Inhibition of EBOV PV with commercially produced EBOV sGP

The effect of in-house co-produced and purified sGP was previously observed to have no effect on PV infectivity (Figure 6.1 and Figure 6.6). To see if the commercially produced sGP had an effect on PV infectivity the experiment was repeated. Infectivity of PV in the presence of sGP; 0.5 µg (experiment 1), 0.03, 0.16 and 0.33 µg (experiment 2) compared to PV alone was analysed with a non-parametric Mann-Whitney test, as the data set did not pass a normality test (Figure 6.10). The analysis revealed no statistical differences between the infectivity of PV alone and PV pre-incubated with sGP.

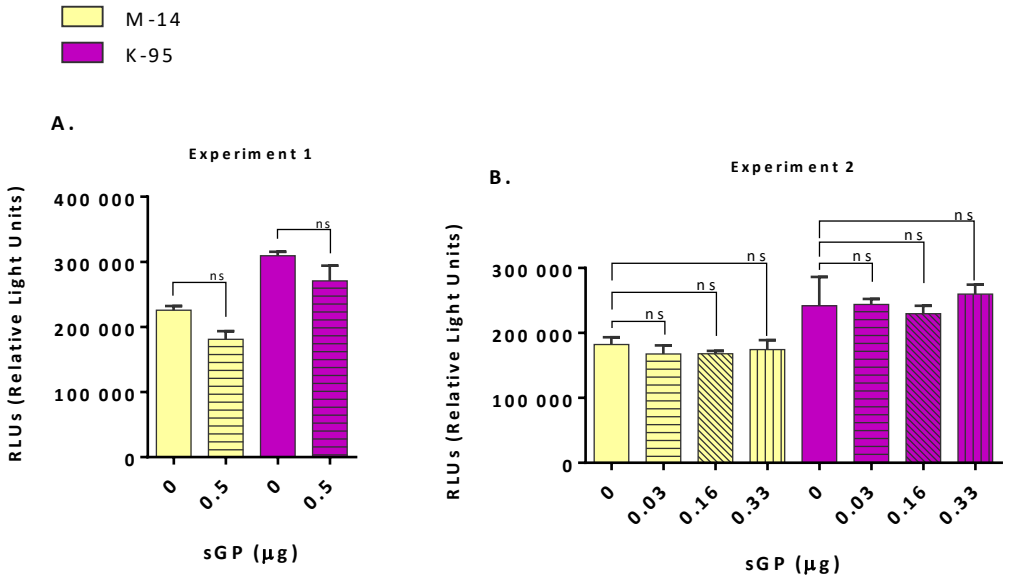


Figure 6.10 Infectivity of EBOV PV incubated with sGP

EBOV PV strains; M-14 and K-95 were incubated with differing amounts of sGP A) 0.5 µg/ well (biological repeat n= 3, technical repeat n= 2) B) 0.03, 0.16 and 0.33 µg/ well (biological repeat n= 2, technical repeat n= 2) prior to infection into TZM-bl cells. Infectivity of PV with sGP was compared to PV alone and a non-parametric Mann-Whitney statistical test was applied, revealing no significant differences. Error bars indicate mean with standard deviation.

6.2.3.4 EBOV PV inhibition with CP in the presence of commercially produced sGP

The impact of sGP on EBOV PV neutralisation with CP was subsequently tested. M-14 and K-95 were incubated with Donor CP samples 49, 7, 15 and 42 alone or in the presence of 0.5 µg sGP. IC_{50} titres for the PV and CP samples, which were determined in previous neutralisation assays, were utilised for this experiment. For all ensuing experiments

inhibition of PV with CP alone or CP together with sGP were calculated relative to EBOV PV neutralisation with EBOV negative human plasma in the presence of sGP (shown as black dotted line in graphs). It was observed for both PV strains A) M-14 and B) K-95 (Figure 6.11) that CP inhibited overall EBOV entry into target cells, however, in the presence of sGP a reversion towards infectivity of PV without CP could be observed suggesting sGP had an impact on CP inhibition of PV.

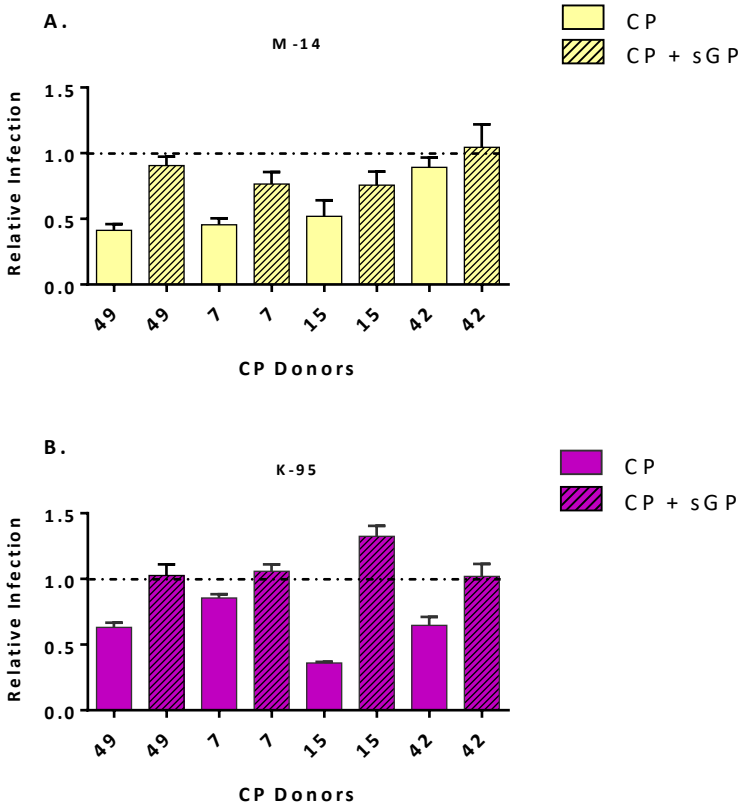


Figure 6.11 IC_{50} CP neutralisation of EBOV PV in the presence of sGP

Neutralisation of A) M-14 and B) K-95 with Donor_CP 49, 7, 15 and 42 was carried out in the presence of 0.5 µg sGP/ well using IC_{50} titres determined from previous neutralisation assays (biological repeat n= 1, technical repeat n= 3). Inhibition of PV with CP (plain bars) and CP in the presence of sGP (lined bars) was plotted in comparison to PV incubated with EBOV negative human plasma and sGP (black dotted lines). Error bars indicate mean with standard deviation.

Following on from this sGP was evaluated for the capacity to increase PV infectivity in the presence of CP. A neutralisation assay was conducted using 10x the quantity of CP Ab titres used in experiment 6.10 for inhibition of the respective PV, either alone or in the presence of 0.5 µg sGP/ well. It was observed with the higher concentration of CP that inhibition of PV

was more pronounced, nonetheless, sGP incubated with CP again revealed an upturn in PV infectivity demonstrating a strong interaction between sGP and CP for both EBOV strains A) M-14 PV and B) K-95 (Figure 6.12).

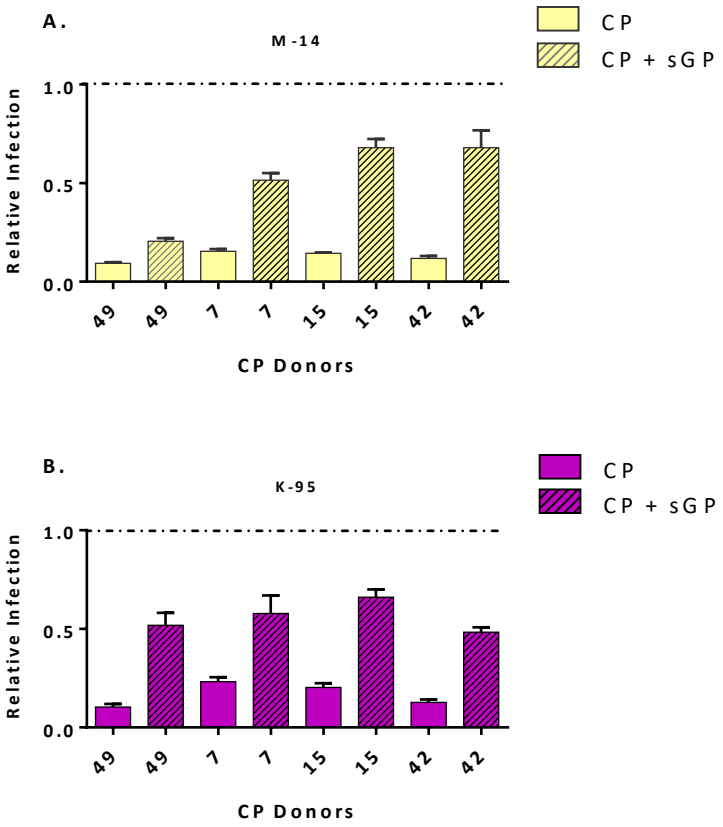


Figure 6.12 10x IC_{50} CP neutralisation of EBOV PV in the presence of sGP

Neutralisation of A) M-14 and B) K-95 with Donor_CP 49, 7, 15 and 42 was carried out in the presence of 0.5 µg sGP/ well using 10x the IC_{50} titre determined from previous neutralisation assay (biological repeat n= 2, technical repeat n= 2). Inhibition of PV in the presence of CP (plain bars) and CP and sGP (lined bars) was plotted in comparison to PV incubated with EBOV negative human plasma and sGP (black dotted lines). Error bars indicate mean with standard deviation.

CP Donor_45, previously observed to have a distinctive neutralisation trend (Discussed in detail 5.2.5), was utilised in a sGP dose dependent experiment. A low neutralising (CP_Donor_45_B) and a high neutralising (CP_Donor_45_D) CP sample from Donor_45 was incubated with M-14 and K-95 PV strains both with and without sGP. Again sGP was seen to reverse the neutralisation effects of CP in all experiments and this was observed to be more prominent in the less potent CP samples A) and C) (Figure 6.13). Collectively, these results

strongly support the concept that sGP can modulate the neutralisation effect of CP on viral infectivity, indicating that sGP can provide immune evasion from Ab responses.

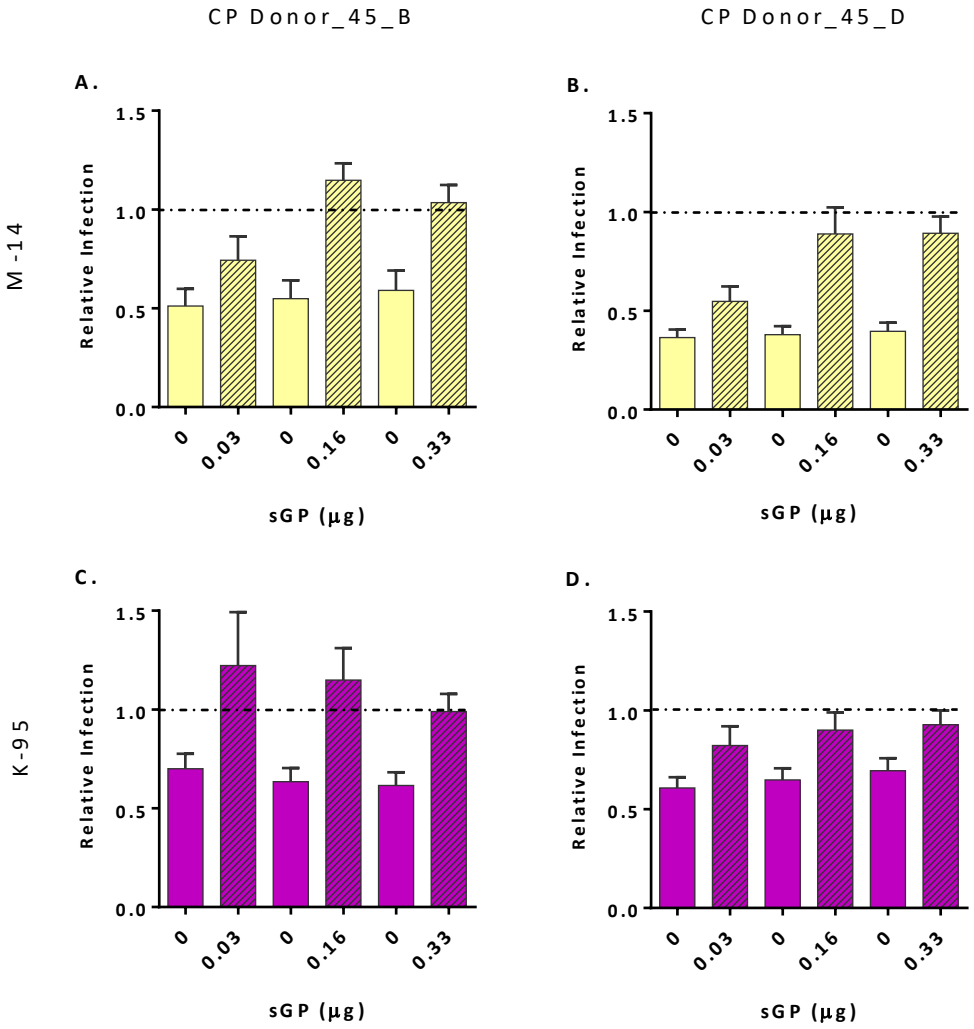


Figure 6.13 IC_{50} neutralisation of EBOV PV with Donor CP_45 in the presence of sGP

Neutralisation of M-14 and K-95 with Donor CP_45 was carried out in the presence of 0.03, 0.16 and 0.33 µg sGP/ well using IC_{50} titres determined from previous neutralisation assay (biological repeat n= 2, technical repeat n= 2). Inhibition of PV in the presence of CP (plain bars) and CP and sGP (lined bars) was plotted in comparison to PV incubated with EBOV negative human plasma and sGP (black dotted lines). Error bars indicate mean with standard deviation.

6.2.3.5 Analysis of sGP reversion of PV infectivity

To further investigate this finding, neutralisation experiments carried out with M-14 and K-95 PV in the presence of sGP was reviewed. The increase in infectivity between CP neutralisation with sGP compared to CP neutralisation alone was calculated as a percentage

increase for the respective EBOV strains. For each donor CP sample the increase in infectivity was plotted for both M-14 and K-95 on an XY graph (Figure 6.14 A). The statistical correlation ($P<0.05$) between M-14 and K-95 data sets revealed that the percentage in increase was similar between the virus strains. In addition the data was plotted as a line graph (Figure 6.14 B) with a non-parametric t-test revealing non-significant differences overall between the two PV strains. However, this analysis highlighted individual differences in sGP reversion which appeared to be neither plasma nor virus dependent. Future research will aim to clarify these individual differences.

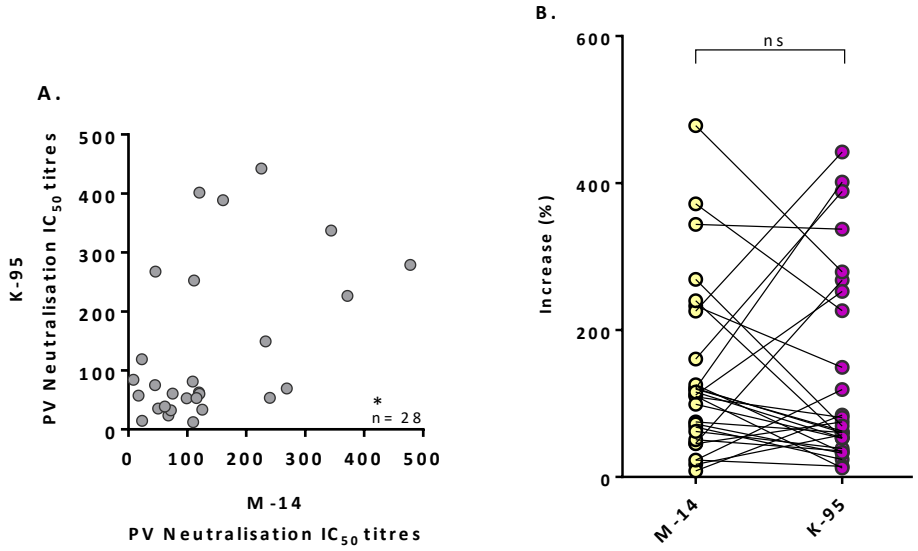


Figure 6.14 Increase in infectivity between PV neutralised with CP and in the presence of sGP

The infectivity difference between PV neutralised with CP alone and CP in the presence of sGP was calculated as a percentage and plotted for M-14 and K-95. A) Correlation statistical analysis ($P<0.05$) was observed between both virus strains. B) A line graph analysed with a Wilcoxon matched-pairs signed rank test showed overall no significant differences between sGP reversion in PV infectivity between M-14 and K-95 virus strains.

6.3 Discussion

Previously (chapters 4 and 5) we investigated the capacity of CP from EBOV survivors to inhibit infectivity of EBOV PV in susceptible cells. We developed upon these findings by aiming to mimic natural EBOV infection where neutralisation would occur in the presence of sGP, the highly abundant EBOV protein primarily produced from infected cells (de La Vega, Wong, Kobinger, *et al.*, 2015). This study was conducted to provide insights into the role sGP plays in PV production, infectivity as well as inhibition with CP.

6.3.1 The potential roles of sGP

6.3.1.1 sGP interaction on PV production and infection

We observed that EBOV PV generated from co-transfection with increasing quantities of sGP required concentrated CP for neutralisation. From a technical perspective it was possible higher amounts of plasmid input during transfection was disruptive to the cellular transcription and translation machinery that produced EBOV GP, which resulted in an altered PV phenotype, thereby, affecting neutralisation of the PV. However, both, infectivity of PV co-produced with different quantities of sGP and the respective p24 production were found to have no statistical differences. Therefore, the observation of decreasing IC_{50} titres with increasing sGP was associated with a biological response. The sGP produced during co-production could have interacted with the EBOV PV or cellular receptors to inhibit virus infectivity, however, sGP appeared to increase virus infectivity in the presence of CP indicating a primary interaction with CP. Furthermore, it was observed that there was no statistical difference between infectivity of PV alone and PV incubated with sGP prior to infection.

6.3.1.2 sGP interaction on PV neutralisation with CP

A comparison between sGP produced during co-production with EBOV PV and in-house produced sGP revealed that the latter was less efficient at interacting with Abs present in CP. It is possible there was a disruption to the sGP during the protein concentration process resulting in less efficient binding of CP to sGP. CP neutralisation of EBOV PV in the presence of commercially produced sGP showed an overall reversion of PV infectivity. This could be attributed to sGP acting as a decoy antigen and adsorbing EBOV specific Abs (Ito et al., 2001) thereby allowing EBOV PV to infect target cells. To explore if this trend was maintained, an sGP dose effect experiment was conducted using much lower sGP concentrations than in

previous experiments. Reversion of PV infectivity was observed with all three sGP quantities tested indicating that even minimal sGP secreted from infected cells of EBOV infected individuals could potentially effect PV inhibition with Abs. Interestingly, it was observed that neutralisation of both M-14 and K-95 conducted with the less potent CP sample (Figure 6.13 A and C) compared to the more potent CP sample (Figure 6.13 B and D), showed a greater reversion of PV infectivity; surpassing infectivity of the negative control in the presence of sGP. The increase in PV infectivity could be explained by a phenomenon known as Ab-dependent enhancement (ADE) of virus infection, whereby virus-specific Abs enhance the entry of virus into target cells (Tirado and Yoon, 2003). The exact mechanism with which this could occur in the presence of sGP needs to be investigated, however, it can be theorised that CP NAbs binding to sGP leaves non-neutralising EBOV specific Abs to bind to EBOV PV permitting ADE or alternatively sGP directly interacts with EBOV PV and CP Abs leading to ADE. It has been documented that human survivor filovirus GP-specific Abs, present at low concentrations, are capable of inducing *in vitro* ADE in target cells (Kuzmina et al., 2018), supporting the possibility that ADE may be responsible for the increase in PV infectivity observed here.

6.3.1.3 Enhanced infectivity of PV in the presence of sGP

The overall correlation between M-14 and K-95 sGP reversion in PV infectivity indicates that the sGP mechanism of interaction with PV is not preferential towards one virus strain. However, when scrutinising individual experiment results; neutralisation of K-95 with Donor CP_45_B (Figure 6.13 C) as well as with a low neutralising CP, Donor CP_15 (Figure 6.11 B) showed a marked increase in PV infectivity of K-95 in the presence of sGP. An increased reversion in PV infectivity observed for K-95 compared to M-14 may be attributed to the Kikwit 1995 strain of the commercially produced sGP. It is possible the sGP interaction through an unknown mechanism with the homologous K-95 EBOV PV, boosts ADE of the PV, permitting increased infectivity. However, further work is required to elucidate if this trend may be substantial. A summary of the outcomes and potential sGP interactions that have been described in this chapter are illustrated (Figure 6.15).

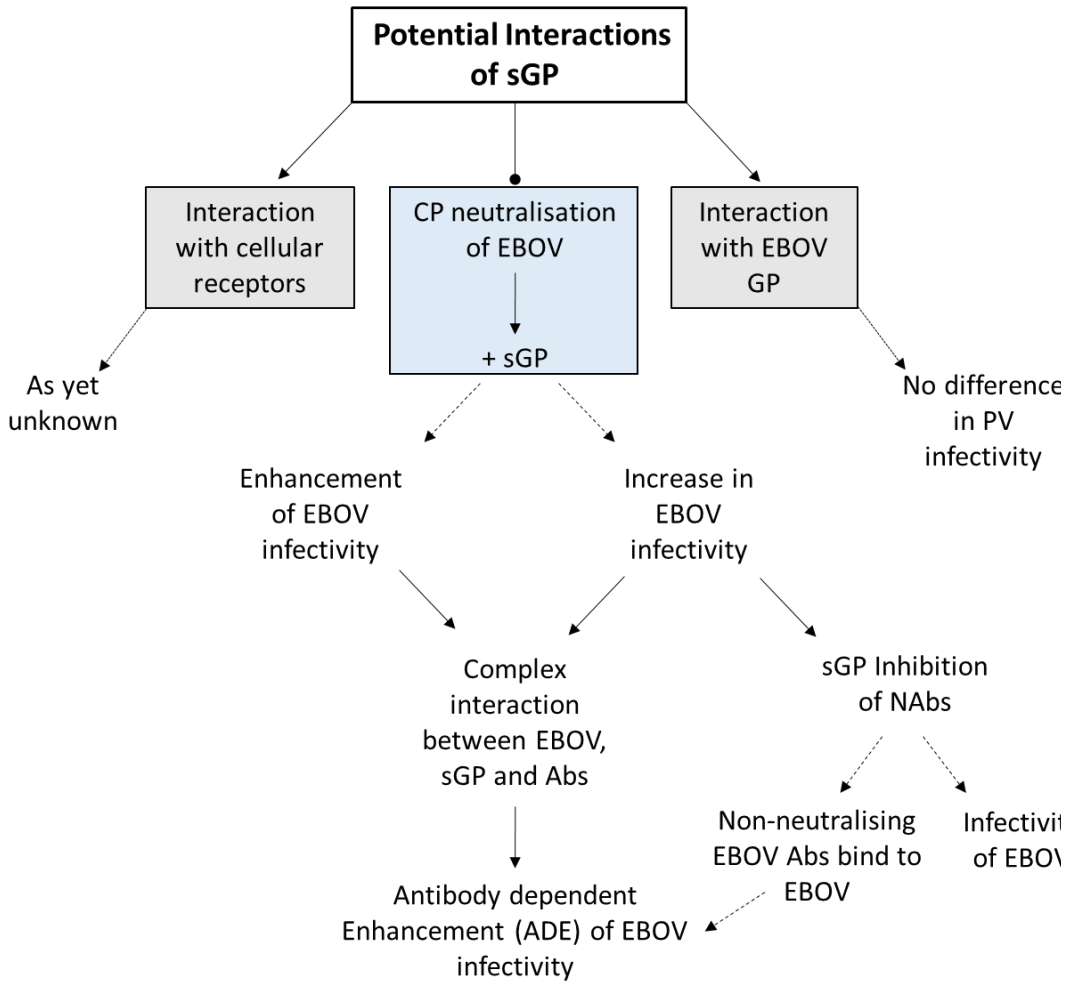


Figure 6.15 Potential interactions of sGP

A summary of sGP interactions with PV and CP NAbs and the potential mechanisms attributed to the findings discussed in this study.

6.3.2 Further work

As discussed previously (1.9.1) transcription of the GP gene can result in ssGP in a minute number of transcripts as well as sGP the primary EBOV protein produced which also yields a cleavage product, Δ-peptide (de La Vega et al., 2015). The different secreted EBOV proteins may all have a specific role which can have different effects in EBOV pathogenesis.

One potential area to be investigated is whether sGP is able to bind to cellular receptors since the Δ-peptide of sGP has been documented to inhibit filovirus entry into target cells (Miller et al., 2012). Here, we have shown sGP has an affinity for Abs present in CP, however, it was

observed that sGP was able to bind to receptors this could alter PV entry into target cells possibly even increasing PV entry via Ab binding.

The preliminary dose effect sGP experiment carried out in this study didn't yield conclusive results on the effect of sGP concentration on the reversion of PV infectivity. Exploring this further could reveal a range at which sGP has more potency in relation to Ab binding.

6.3.3 Chapter conclusion

The work presented in this chapter deduced that co-production and co-infection of sGP with EBOV PV did not alter PV production nor interact significantly with EBOV to alter infectivity of the virus. However, it was observed that sGP was able to revert PV infectivity in the presence of CP NAbs, possibly through sGP acting as a decoy antigen adsorbing CP NAbs or alternatively through the complex mechanism of ADE. Nonetheless, this is a previously undocumented finding and illustrates a potential new role for sGP in EBOV pathogenesis.

7 Final Summary and Conclusion

7.1 Key Findings

7.1.1 Results One: Establishing and optimising an Ebola pseudo-typed virus assay

1. A robust EBOV PV assay comprising of a HIV-1 backbone complemented with an EBOV GP were generated and studied.
2. A range of EBOV GP envelope input was identified which revealed similar infectious titres and was reflected in p24 quantitation.
3. Implementation of a PV infectivity exclusion criteria ensured respective strains of PV stocks had comparable infectious titres.
4. An EBOV negative plasma control range permitted individual assay validation.
5. Our study was validated against the NIBSC international reference standards for the assessment of EBOV specific Abs.
6. Different batches of EBOV PV production did not affect repeated neutralisation assay titres.

7.1.2 Results Two: A cross-sectional analysis of neutralising Abs of Ebola survivor convalescent plasma

1. Wide-ranging neutralisation capacity of EBOV CP NAbs between EBOV survivors.
2. Overall no neutralisation differences by CP NAbs between the 2013 epidemic, 1995 Kikwit and the 2013 mutant strains.
3. Gender, age, BMI and disease severity did not statistically influence NAb titres in this cohort.
4. A trend was observed between increased disease severity and a reduction in CP NAbs.
5. Donor sampling time did not associate with CP NAb titres.

7.1.3 Results Three: Longitudinal analysis of neutralising Abs from convalescent plasma of Ebola survivors

1. Identified diverse donor specific CP NAb profiles.
2. Three independent assays: PV neutralisation assay, ELISA and PRNT all corroborated with the measured CP NAb titres of the EBOV survivors.

3. Donor_45 observed a steep boost in CP NAb titres and Abs against NP and VP40, which was attributed to a strong EBOV antigenic stimulation.
4. Boosted CP NAbs of Donor_45 showed differential neutralisation of the 2013 EBOV strain compared to the mutant and 1995 strain.
5. Mathematical analysis deduced a growth and decline rate for CP Abs.

7.1.4 Results Four: EBOV soluble glycoprotein interaction with neutralising Abs of Ebola survivor convalescent plasma

1. sGP did not affect PV production or infectivity.
2. sGP was able to revert PV infectivity in the presence of CP NAbs.
3. sGP enhanced reversion of PV infectivity was observed in the presence of low neutralising CP NAbs.
4. sGP reversion of PV infectivity was not statistically different between EBOV strains.

7.2 Implications of findings

7.2.1 Assay Development

7.2.1.1 PV strains

For this study two distinct EBOV isolates were chosen for analysis, the 2013 Makona epidemic strain and the 1995 Kikwit strain. The 2013-2016 epidemic strain was used to assess the neutralisation capacity of the homologous EBOV CP. To determine how effectively the 2013-2016 CP would fare neutralising previous circulating EBOV strains the 1995 Kikwit GP was selected, the strain was utilised in the ring vaccination study using vaccine V920 (Henao-Restrepo et al., 2015). A comparison between the 2013 and 1995 strains indicated that the elicited NAbs in our CP donors could effectively inhibit a strain introduced 20 years earlier into the population.

At the beginning of this study the extent of virus evolution was unknown, therefore, we designed a plasmid expressing an EBOV GP comprising of early mutations that appeared during the epidemic in order to try mimic virus escape mutations. Escape mutations would be selected amino acid alterations which resist Ab-mediated neutralisation (Payne, 2017). This was utilised to assess the extent to which CP NAbs were able to inhibit virus.

7.2.1.2 Wider use of PV assay

PV assays have been well utilised for characterisation of emerging viruses that affect humans including the study of EBOV. A comparative study which assessed ELISA, PV and WT EBOV Ab quantitation assays concluded that many were not fit for purpose and that the WT compared to the PV assays predominantly gave credible results, they also concluded that the assay platform selected should be chosen with care to ensure suitability (Wilkinson et al., 2017). Although there are shortfalls with the PV assays including the possible generation of false positives or differential sensitivity capacity depending on the PV system used (Lambe et al., 2017). Here we demonstrated that our PV assay was suitable for the evaluation of EBOV NAbs. Neutralisation with the EBOV International reference reagents were comparable to a PV assay utilised in the NIBSC study; which was observed to have a strong correlation with the reference WT virus (Wilkinson et al., 2017). This correlation was further iterated between our PV assay and the RCE PRNT in our study, thus indicating our assay was operating similar to the 'gold' standard assay for EBOV NAb assessment.

It has been stated that new filovirus PV assays require stringent standardisation using validated negative and positive controls (Lambe et al., 2017). In our study we considered previously undocumented parameters that could impact PV production which was evaluated through rigorous control experiments. The controls not only aided in the development of our EBOV PV assay but can be applied for the systematic production of other virus types. The diversity of emerging infectious disease epidemics requires the rapid implementation of the PV system for use in place of highly pathogenic viruses, the work documented here illustrates a comprehensive list of parameters that should be explored when producing PV and which may impact on the overall robustness of the assay.

The robustness of a PV assay is important in the development of quantitative platform technologies that can be utilised for diagnostic purposes as well as in the evaluation of phase I clinical studies, to assess both the quantity and potency of the antigen-specific Ab response of immunological therapeutics such as vaccination, CP and immunoglobulins (Lambe et al., 2017; Wilkinson et al., 2017).

7.2.2 Ab responses

This thesis documents a comprehensive study describing human CP NAb titres in natural EBOV infection.

7.2.2.1 Diverse EBOV NAb titres

The diverse NAb titres observed between EBOV survivors within this cohort were not attributed to the time post convalescence, nor to donor parameters such as sex, age, BMI or disease severity. However, it was observed that with increased disease severity, NAb titres decreased which could be associated with a higher viral load. Viral load is an important indicator of fatality rate, it has been documented that a higher viral load and a more severe condition at the time of patient admission, were independently associated with an outcome of death in EVD patients (Ji et al., 2016; Mobula et al., 2018). Although the EVD patients in this study were survivors it is plausible that a higher viral load correlates with increased disease severity and subsequent impairment of the immune system, hence lower induction of efficacious EBOV NAb titres. Unfortunately, donor clinical data including patient viral loads at time of EBOV infection was not accessible and therefore could not be used to provide more insightful analyses between clinical parameters and CP NAbs titres.

7.2.2.2 Low/ non-NAb titres

Low or non-NAbs detected in CP with our assay suggested that for some survivors their NAb titres may have decreased rapidly at the onset of the convalescent period or alternatively NAbs may not have been a crucial aspect of immunity responsible for these survivors overcoming EBOV. An early controlled inflammatory response initiated by the innate immune system has also been associated with a recovery from EBOV (Olejnik et al., 2011; Wong et al., 2014). Although feasibility would have been difficult, a comparison of Ab titres and clinical presentations at endpoint (recovery or death) may have been insightful for this study in order to gain a more accurate perception of Ab levels associated with overcoming EBOV infection. Studies that compared patients till endpoint have been able to deduce statistical associations between EVD characteristics and patient outcome (Ji et al., 2016).

The remarkable EBOV NAb profile of Donor_45 illustrated the importance of circulating NAb titres in convalescent individuals. It is highly improbable that the rapid boost in EBOV specific NAb responses observed could be attributed to any reason other than an EBOV antigenic stimulation. This donor revealed interesting findings: the first set of CP samples from

Donor_45 had low NAb titres, indicating that individuals from the cohort who were deemed to have low CP neutralisation against EBOV may in fact still elicit a strong immune response conferring protection, when faced with an EBOV antigenic stimulation.

Following the 2013-2016 epidemic, persistence of EBOV in survivors has been well documented in bodily fluids and immune privileged sites after EBOV RNA was non-detectable in blood (Deen et al., 2017; Varkey et al., 2015). Here we observe the case of an asymptomatic donor who showed strong indications of an EBOV antigenic stimulation of the immune system. This is more likely attributed to EBOV residing in an immune privileged site as opposed to an EBOV infection from the external environment, as the donor tested EBOV RNA blood negative. For this reason it can also be speculated that EBOV expressed proteins as opposed to active virus has been secreted into the periphery provoking the immune system. Alternatively, small pockets of viral activity within a sequestered anatomical site may have occurred and which was adequately controlled by effective immune responses. It has been documented in EVD patients treated in Emory University hospital that plasmablast population peaks coincided with the production of EBOV specific IgG and that EBOV specific plasmablasts were detected in the blood even 2 months after symptom onset (McElroy et al., 2015), pointing towards EBOV survivors having a robust humoral immune response.

7.2.2.3 Increasing/ decreasing NAb titres

Increases in EBOV Ab responses were observed for other survivors, and in some an increasing/ decreasing NAb trend was observed in donors who provided longitudinal samplings. This raises several questions: i) whether donors are continuously subjected to EBOV antigenic stimulations, possibly from virus residing in immune privileged sites which causes increased and decreased NAb trends ii) If donors are harbouring virus in immune privileged sites, what is the event that causes active virus to be produced and released into the periphery. Answering these questions will help in understanding the Ab responses that curtail active EBOV infection.

A mathematical analysis was used to calculate the overall CP NAb growth and decline rate of donors who showed increases and decreases in their NAb titres, this pointed towards a scenario where a survivor's immune system provoked with EBOV antigen produced NAbs quicker than the time in which the Abs decreased. This self re-stimulation of the immune response can therefore keep NAb titres at sufficient levels where control of emerging virus

can be adequately controlled. The concern here is that if NAb levels fall below a sufficiently controlling threshold then the reactivation of viral expression may therefore no longer be sufficiently neutralised and an uncontrolled infection may result. Given timings of the last epidemic and the time that has passed it may have been expected that this would have been documented. Countering this, some studies have shown that transmission of the virus has occurred up to a year after clearance which does suggest that reactivation can occur and that induced immune responses are not successfully clearing the virus. Time will ultimately tell whether indeed this is the case and to what extent an Ebola survivor can experience re-activation.

7.2.3 EBOV Vaccination

7.2.3.1 V920 trial

It has been reported that participants taking part in the trial for the EBOV vaccine candidate V920 maintained overall Ab titres targeting EBOV, however, NAb titres were low and declined rapidly (Bornholdt and Bradfute, 2018). The EBOV vaccine targeted the trimeric EBOV surface GP which facilitates viral entry (Bornholdt and Bradfute, 2018), in this study we demonstrated the association between levels of CP NAb and the efficiency with which EBOV is inhibited. Our research would imply that if the trial participants were challenged with EBOV their low NAb levels may not confer protection. However, it is important to note that the differential roles of non-neutralising and NAbs targeting EBOV have not been clearly defined, although NAbs are a key determinant in infection control, non-NAbs may also play an important role in curbing infection. Therefore the long-term immunity provided to the vaccinees against EBOV will need to be monitored (Bornholdt and Bradfute, 2018; Lambe et al., 2017; Su and Moog, 2014).

The various phase I clinical trials of vaccines against EVD, utilised different strains of EBOV in their composition (Lambe et al., 2017). In this study we showed that CP NAbs elicited against the 2013 Makona EBOV variant exhibited equivalent potency in neutralising the EBOV PV strain bearing the Kikwit 1995 GP. This indicates that an EBOV strain which came into circulation approximately 20 years prior to the epidemic strain was successfully neutralised by heterologous CP NAbs to the same extent as the homologous strain. This bodes well for individuals who produce Abs in response to one strain of EBOV vaccine; they are likely to have a broad protection if challenged with a variant of EBOV different to the vaccine strain.

Additionally, it suggests that a vaccine based on the 2013 epidemic strain may still confer overall protection against yet undocumented EBOV strains.

In this study we looked at the ability of CP NAbs to inhibit a predicted EBOV escape mutant. Here, we observed overall no comparable CP inhibitory differences between the 2013 mutant and the epidemic strain. However, the boosted NAbs of Donor_45 was observed to have differential neutralisation potential between the 2013 epidemic strain and the 1995 and mutant strains. This suggests that overall Abs produced as a result of vaccine administration would be able to broadly protect a population against EBOV, potentially conferring protection even when challenged with a mutant strain. However, it is possible that EBOV variants will arise which are less neutralisable and will reduce the effectiveness of vaccines. A 'watch' list of EBOV mutations was developed, if mutations from the list arose during an epidemic they would warrant close attention as these mutations may reflect the evolutionary response of EBOV which might compromise vaccine efforts (Miller et al., 2016).

7.2.4 sGP Interactions

It has been previously documented that sGP may act as a decoy antigen to Abs targeting the EBOV GP (Ito et al., 2001). The results in this study demonstrated the ability of sGP to revert the infectivity of EBOV PV in the presence of CP NAbs, which may have consequences with regard to EBOV therapeutic prevention.

A possible scenario where sGP may have an effect is when an individual administered with an attenuated EBOV vaccine would elicit a humoral response and produce Abs against the EBOV GP. If the person was challenged with an EBOV infection these Abs would target EBOV and neutralise the virus. However, it is possible in this scenario that sGP produced from EBOV infected cells could serve as a decoy antigen (Ito, Watanabe and Takada, 2001) and adsorb the EBOV GP targeting Abs, leaving EBOV uninhibited to infect host cells. Likewise, sGP could interact with EBOV and EBOV specific Abs through an as yet unconfirmed mechanism; likely, ADE to increase EBOV infectivity (Kuzmina et al., 2018). From results indicated in this study, the increase in EBOV infectivity could potentially be higher if the EBOV strain was homologous to the Abs elicited by the vaccine. The EBOV sGP could potentially render prophylactic Ab based therapeutics less effective against EBOV. Although further investigation is required studying the precise roles of sGP in pathogenesis, production during EBOV infection is a factor that needs to be considered during EBOV therapeutic design. The

data shown here indicates sGP is an important protein to be considered during EBOV infection and is an important factor to take into account when designing new and improved vaccines and therapeutic products (de La Vega, Wong, Kobinger, *et al.*, 2015).

7.2.5 Clinical Implications

As discussed previously NAb titres are not the only aspect of immunity to aid in overcoming EBOV infection, however they are involved. The half-life of NAbs calculated in this study indicates the possible benefits of boosting Ab titres via vaccination for EBOV survivors. A nationwide vaccine boosting strategy requires funding as well as in depth political and technical (WHO, 2014; PAHO, 2010) assessments prior to implementation of the vaccine strategy, which may prove challenging in resource constrained settings. Therefore, a compromise could be to implement vaccine boosts for health care workers, as they are at higher risk of contracting EBOV, as was seen in the 2013 epidemic. By May 2015, 1.45%, 8.07% and 6.85% of healthcare workers compared to 0.02%, 0.11% and 0.06% of the general population had died in Guinea, Liberia and Sierra Leone, respectively. The disproportionate deaths of health care workers left skill shortages in countries that had few health personnel to begin with (Evans et al., 2015). Since the epidemic, EBOV outbreaks have continued as seen in the current DRC outbreak which started in May 2018 (MSF, 2018) and with the frequency of EBOV outbreaks expected to be continuous, the protection of health care workers should be considered foremost in order to maintain healthcare infrastructure in these countries.

7.3 Prospective further work

The work presented in this thesis has shown a high degree of EBOV neutralisation capacity of plasma from individuals recovering from Ebola. We have shown overall no differences in the CP neutralisation capacity against three PV EBOV strains (M-14, K-95 and MEM-14). However, differences were seen with Donor_045, where boosted Ab responses showed less neutralisation capacity to M-14 than against either the MEM-14 or K-95 strain. To further understand these differences epitope mapping of the EBOV PV strains recognised by CP could be conducted. This would elucidate CP neutralising epitopes which are shared or are different between the PV strains giving further insight into the level of cross-protection offered by CP as well as indicating any shared neutralising epitopes with mAbs (discussed in Table 3.3). Following CP epitope mapping molecular modelling could be utilised to estimate

stability changes in mutations and therefore a 'watch list' of potential EBOV escape mutations based on CP neutralisation could be produced (Miller et al., 2016).

During the 2013-2016 EBOV epidemic the predominant trial; Ebola-TX, assessed the therapeutic benefits of CP donated from EBOV survivors to those suffering from EVD. The study concluded that overall, CP did not confer any survival benefits to an EVD sufferer, however, the key caveat was that neutralisation titres of the CP were unknown prior to transfusion. Within our cohort we have shown the wide-ranging neutralisation capacity of CP, it is possible that individuals with higher neutralising CP may confer a survival advantage compared to those with lower neutralising CP and this is an avenue that could still be explored with regards to Ebola therapeutics.

The scale of the EBOV epidemic in West Africa allowed many experimental drugs and therapies to be accelerated for use by the WHO on the basis of compassionate grounds (Espeland et al., 2018). The purpose was to potentially have a direct impact on the continuing outbreak as well as assessing efficacy and clinical benefit of the therapies or drugs (Espeland et al., 2018). The V920 vaccine bearing the 1995 Kikwit immunogen has been the predominantly administered anti-EBOV vaccine during the epidemic (Henao-Restrepo et al., 2015). To further understand neutralising epitopes of EBOV it would be of interest to assess the neutralising repertoire of EVD convalescent individuals in comparison to those who received the V920 vaccine. This would give an indication as to the potency of EBOV NAbs produced in natural immunity compared to those with passive immunity to neutralise EBOV and also highlight the neutralising EBOV epitopes that are shared or are different between the two different types of immunities studied. This work would contribute to understanding and producing efficacious anti-EBOV vaccines.

Donors providing multiple CP samples over a period of time enabled the longitudinal assessment of CP NAbs in this thesis. Scrutinising the NAb responses of individual donors highlighted the different longitudinal responses that could be observed. To build upon the longitudinal data of this study and to continue to gain insights into the long term NAb responses of EBOV survivors it would be insightful to follow up on the survivors and acquire further plasma samples. This study would allow the assessment of whether NAbs against EBOV can be retained 3-3.5 years after the individual's last plasma sample donated. Regular CP donations over a period of a year would indicate if survivors who previously showed high

neutralising titres still retained these titres and also would show if increasing/ decreasing neutralising trends were still prominent in the cohort.

The primary protein produced from EBOV GP transcripts is sGP; a secreted protein which is produced from EBOV infected cells and is found in abundance in those suffering from EVD. The potential roles of sGP in EBOV pathogenesis have been proposed (discussed in 1.9.2.1) however, to date further research is still required to support these roles. This thesis aimed to describe the role of sGP in the presence of CP by mimicking natural EBOV infection, where neutralisation of EBOV would occur in the presence of sGP. Our results showed an interaction between sGP, PV and CP, which resulted in the reversion of PV infectivity compared to PV which was incubated with CP alone. To identify and characterise the mechanism of sGP interaction with PV and CP NAbs a possible research approach that could be undertaken is understanding the interaction of sGP with cellular receptors. It has been documented that the sGP cleavage product, Δ-peptide is capable of binding to receptors on filovirus permissive cells thereby preventing filovirus entry (de La Vega, Wong, Kobinger, *et al.*, 2015). Although not documented for sGP, this role is possible and could be involved in preventing or limiting EBOV infection of all target cells. Our results also pointed towards a higher reversion of infectivity between homologous EBOV PV strains and sGP. To further investigate this, experiments previously conducted (6.2.3) could be repeated with sGP; Makona 2013 strain to study if there was any significance in PV reversion with regards to this parameter, which as described earlier (7.2.4) may have implications in the efficacy of prophylactic treatment.

7.4 Overall summary

This thesis describes the development of a well characterised EBOV PV neutralisation assay with which previously undocumented control measures assessed here, were shown to effect PV production and infectivity. These control measures could be implemented in the systematic characterisation of any new PV systems. We have also shown that the measurements of neutralisation correlates with both total Ab responses against EBOV as well as neutralisation of replication competent virus. The PV neutralisation assay permitted for a novel and comprehensive analysis of naturally occurring EBOV NAbs in a cohort of convalescent survivors from the 2013-2016 epidemic. The breadth and specificity of the NAb immune responses were evaluated from both a cross-sectional and longitudinal perspective. There was found to be a wide-range of neutralisation potential between CP samples from

survivors which were found to be consistent in neutralising three different strains of GP tested, representing recent as well as older strains. This result indicates that immune responses induced against a specific EBOV strain will in all likelihood possess good neutralisation potential against unrelated strains. One of the most striking findings is the high degree of Ab boosting observed in recovering individuals of our cohort. This boosting indicates that periodic antigenic re-stimulation is common in recovering patients with consequences for both disease and re-seeding of infection. Mathematical modelling revealed both the decreases; half-life for EBOV Ab decay as well as the increases; doubling time upon re-stimulation, for the Ab responses assessed in this study. Noticeably there is an on-going viral re-activation which modulates induced immune responses in recovering patients and which provides an indication of what is required to be induced in vaccine recipients in order to either control viral re-emergence or prevent subsequent infection. The sGP interaction with PV and CP NAbs revealed a previously undocumented finding, that sGP was not only able to hinder CP neutralisation of PV but also enhance PV infectivity. Furthermore, elucidation of these EBOV protein interactions will lead to the development of improved prophylactic approaches intended to limit the detrimental impact of EBOV pathogenesis. Given the nature of this virus and its continual re-emergence either at the population level or within survivors, new and improved strategies aimed at curtailing infection would be highly desirable and beneficial to global health.

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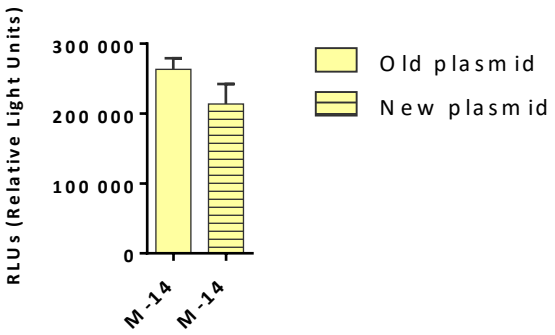
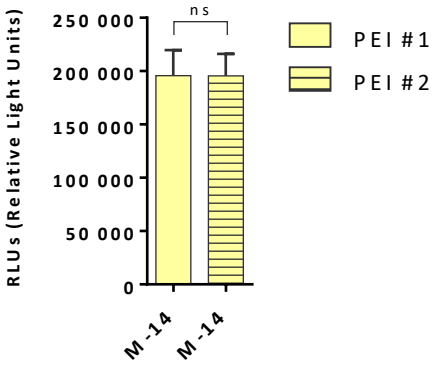
Appendix



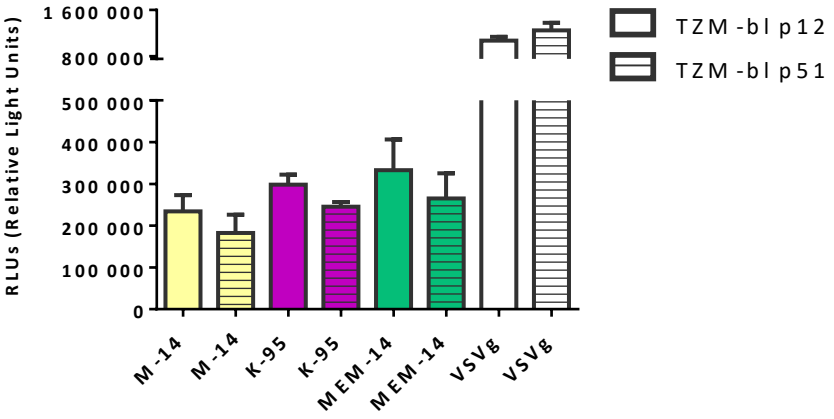
Appendix 1 Plasmid stock on PV infectivity

M-14 PV was produced with previously utilised and newly prepared plasmid stocks. PV infectivity between the two stocks was compared in TZM-bl cells. The data set was too small to yield statistical significance.



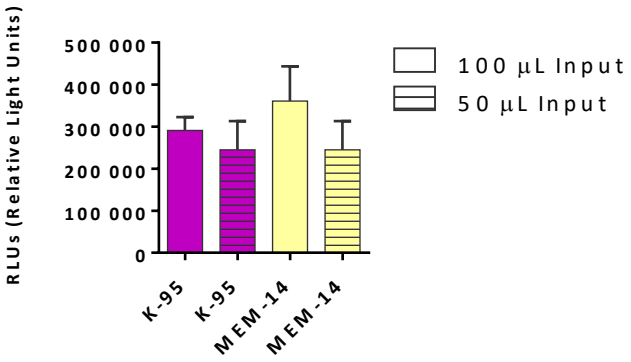
Appendix 2 Variant PEI batches on PV infectivity

M-14 PV was produced with previously utilised and newly prepared PEI transfection reagent. PV infectivity between the two stocks was compared in TZM-bl cells. A non-parametric t-test revealed no significant differences between the two stocks.



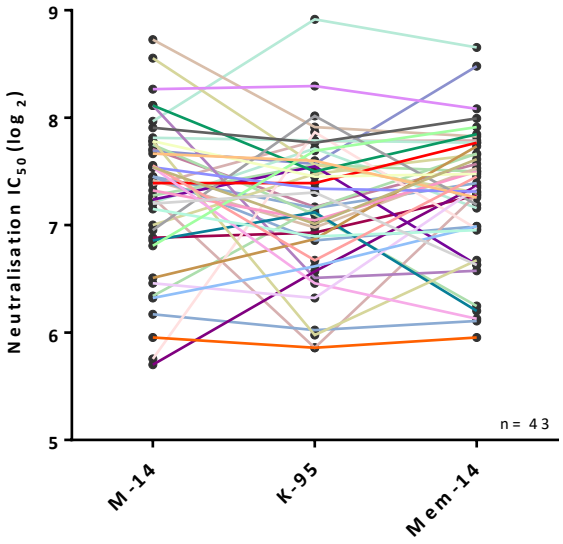
Appendix 3 Cell passage number on PV infectivity

TZM-bl cells p12 and p51 were compared for PV infectivity of EBOV PV strains M-14, K-95, MEM-14 and VSVg. Data set was too small to yield any statistical differences.



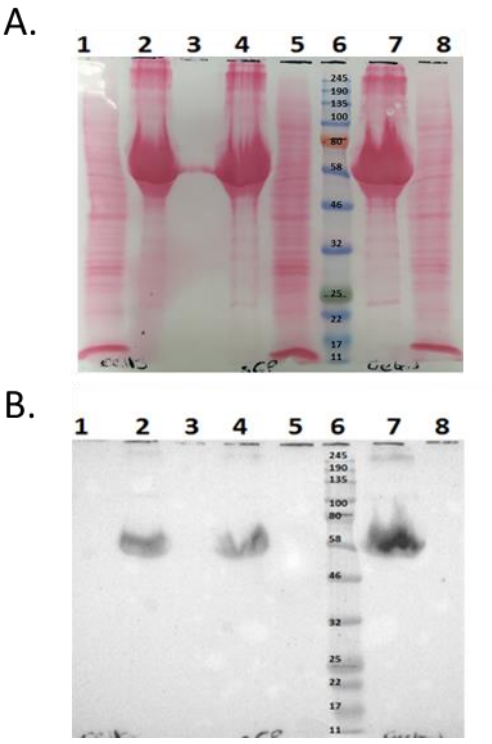
Appendix 4 Virus volume on PV infectivity

PV infectivity of 100 µl and 50 µl virus input during infection were compared in TZM-bl cells in both K-95 and MEM-14 PV strains. 50 µl PV input yielded less infectious titres than 100 µl input. Data set was too small to yield any statistical differences.



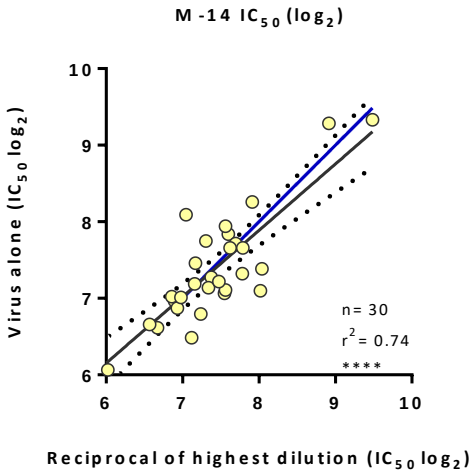
Appendix 5 Individual CP comparison between EBOV strains M-14, K-95 and MEM-14

Neutralisation IC_{50} titres obtained from M-14, K-95 and MEM-14 with CP (n= 43) were plotted to compare IC_{50} titres of individual CP samples between the three EBOV strains. Each colour represents an individual CP sample.

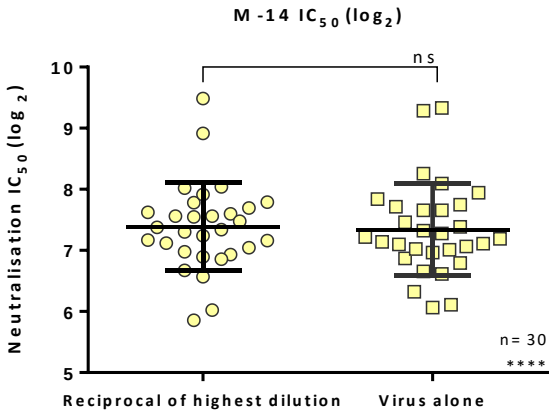


Appendix 6 Ponceau red stain and Immunoblot

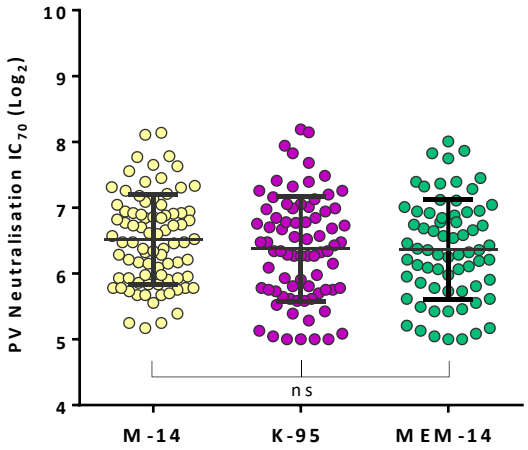
Detection of in-house produced sGP (lane 4) and M-14 PV (lane 7) alongside the negative control; supernatant of transfected cells alone (lane 2). A) Ponceau stained proteins, post membrane transfer shows abundant protein in lanes 4 and 7 as well as negative control. B) FE25 (ENZO) anti-EBOV GP Ab probed membrane shows non-specific binding to protein present in the supernatant (likely FBS).



Appendix 7 Correlation between neutralisation IC50 titres deduced using two methods
Neutralisation IC_{50} titres measured from CP inhibition (n= 30 donors) of the M-14 EBOV PV were deduced using two methods. M-14 neutralisation was either compared to the uninhibited infectivity of M-14 PV alone or as a reciprocal of the highest dilution, which was deemed a non-neutralising dilution. Neutralisation IC_{50} titres deduced using both methods were scrutinised using a non-parametric Spearmann correlation analysis, revealing statistically strong ($r^2 = 0.74$) significant correlations (**** $p<0.0001$) between both sets of IC_{50} titres. Black line indicates slope and blue line indicates the perfect line.



Appendix 8 Comparison between neutralisation IC50 titres deduced using two methods
Neutralisation IC_{50} titres measured from CP inhibition (n= 30 donors) of the M-14 EBOV PV were deduced using two methods. M-14 neutralisation was either compared to the uninhibited infectivity of M-14 PV alone or as a reciprocal of the highest dilution, which was deemed a non-neutralising dilution. Neutralisation IC_{50} titres deduced using both methods were scrutinised using a Wilcoxon matched-pairs signed rank test, revealing a strong probability (****$P< 0.0001$) of no statistical significance between the IC_{50} values of both data sets. Error bars indicate mean with standard deviation.



Appendix 9 Comparison of CP NAbs IC_{70} titre of K-95, M-14 and MEM-14

Neutralisation IC_{50} titres obtained from M-14, K-95 and MEM-14 with CP were analysed by Kruskal-Wallis test yielding no significant differences.

Appendix 10 Publication utilising data arising from this PhD.

Richard Tedder, Dhan Samuel, Steve Dicks, Janet T Scott, Samreen Ijaz, Kitty Smith, **Charlene Adaken**, Christine Cole, Samuel Baker, Tansy Edwards Philip Kamara, Osman Kargbo, Saidia Niazi, Davis Nwakanma, Umberto d'Alessandro, Graham Burch, Heidi Doughty, Nick Andrews, Judith Glynn, Johan van Griensven, Ebola CP Consortium Investigators, Georgios Pollakis, William Paxton, and Malcolm Semple. 'Detection, characterisation and enrolment of donors of Ebola convalescent plasma in Sierra Leone'. *Transfusion*, 58(5), pp. 1289–1298. doi: 10.1111/trf.14580.