Molecular Immunology 48 (2011) 2027–2037

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Isolation and characterisation of *Ebolavirus*-specific recombinant antibody fragments from murine and shark immune libraries

Sarah A. Goodchild [a,*], Helen Dooley [b], Randal J. Schoepp [c], Martin Flajnik [b], Stephen G. Lonsdale [a]

[a] *Defence Science and Technology Laboratory, Porton Down, Salisbury SP4 0JQ, UK*
[b] *Department of Microbiology and Immunology, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, Maryland 21201, USA*
[c] *Diagnostic Systems Division, United States Army Research Institute of Infectious Diseases, Ft Detrick, Maryland 21702, USA*

ARTICLE INFO

Article history:
Received 8 February 2011
Received in revised form 9 June 2011
Accepted 13 June 2011
Available online 12 July 2011

Keywords:
Nurse shark
scFv
IgNAR
Ebolavirus
Bio-panning

ABSTRACT

Members of the genus *Ebolavirus* cause fulminating outbreaks of disease in human and non-human primate populations with a mortality rate up to 90%. To facilitate rapid detection of these pathogens in clinical and environmental samples, robust reagents capable of providing sensitive and specific detection are required. In this work recombinant antibody libraries were generated from murine (single chain variable domain fragment; scFv) and nurse shark, *Ginglymostoma cirratum* (IgNAR V) hosts immunised with *Zaire ebolavirus*. This provides the first recorded IgNAR V response against a particulate antigen in the nurse shark. Both murine scFv and shark IgNAR V libraries were panned by phage display technology to identify useful antibodies for the generation of immunological detection reagents. Two murine scFv were shown to have specificity to the *Zaire ebolavirus* viral matrix protein VP40. Two isolated IgNAR V were shown to bind to the viral nucleoprotein (NP) and to capture viable *Zaire ebolavirus* with a high degree of sensitivity. Assays developed with IgNAR V cross-reacted to *Reston ebolavirus*, *Sudan ebolavirus* and *Bundibugyo ebolavirus*. Despite this broad reactivity, neither of IgNAR V showed reactivity to *Côte d'Ivoire ebolavirus*. IgNAR V was substantially more resistant to irreversible thermal denaturation than murine scFv and monoclonal IgG in a comparative test. The demonstrable robustness of the IgNAR V domains may offer enhanced utility as immunological detection reagents in fieldable biosensor applications for use in tropical or subtropical countries where outbreaks of Ebolavirus haemorrhagic fever occur.

1. Introduction

The genus *Ebolavirus* is comprised of four recognised species namely, *Zaire ebolavirus*, *Sudan ebolavirus*, *Reston ebolavirus* and *Côte d'Ivoire ebolavirus* (ICTV, 2005) with a putative fifth species of *Ebolavirus* (*Bundibuygo ebolavirus*) isolated in western Uganda (Towner et al., 2008). *Ebolavirus* species are the causative agent of Ebolavirus Haemorrhagic Fever (EVHF). However, only *Zaire ebolavirus* and *Sudan ebolavirus* and more recently *Bundibuygo ebolavirus* have been associated with large sporadic outbreaks of disease in sub-Saharan Africa with mortality rates ranging between 36% and 90% dependent on species (Sanchez et al., 2007; Towner et al., 2008). In addition to the substantial public health concern associated with natural outbreaks of Ebolavirus-associated disease, public domain information pertaining to the weaponisation programme of the Former Soviet Union (FSU) (Alibek, 2000) indicate that Ebolaviruses have been considered by several groups to be a potential agent of biological warfare and bio-terrorism (Borio et al., 2002; Broussard, 2001; CDC, 2000). The severity of natural and potential deliberate outbreaks of EVHF in combination with the current lack of licensed vaccine or post exposure therapies places emphasis on the development of robust assays to determine the presence of *Ebolavirus* species. Early detection and diagnosis is critical to instigating practices such as barrier nursing and isolation of suspected cases that are essential in preventing wide scale spread of the virus and infection of medical staff and care givers (Khan et al., 1999; Lloyd et al., 1999).

Where conditions allow, the amplification and detection of genomic nucleic acid material from *Ebolavirus* species is a powerful tool for laboratory diagnosis (Grolla et al., 2005; Towner et al., 2004; Rodriguez et al., 1999; Leroy et al., 2000). However, the genetic divergence between Ebolavirus strains may mean that highly specific assays may not be resilient to emergence of new strains and species (Towner et al., 2008). Alternatively, immunoassays are available that screen for the presence of Ebolavirus-specific antibodies in serum samples (Ksiazek et al., 1999b; Merzlikin et al., 1995; Rowe et al., 1999; Groen et al., 2003; Prehaud et al., 1998; Saijo et al., 2001; Georges-Courbot et al., 1997). However, given

* Corresponding author at: Tel.: +44(0)1980 614838; fax: +44(0)1980 613987.
E-mail address: sagoodchild@mail.dstl.gov.uk (S.A. Goodchild).

0161-5890/$ – see front matter.
doi:10.1016/j.molimm.2011.06.437

that the humoral immune response only becomes quantifiable late in the disease course (Merzlikin et al., 1995; Rowe et al., 1999), the benefit of such assays as field diagnostics has been questioned (Ksiazek et al., 1999a). Antibodies are also available for the direct detection of Ebolavirus-specific antigens from environmental samples and/or patient sera. These include polyclonal sera from a variety of animals (Chepurnov et al., 1994; Ksiazek et al., 1992; Merzlikin et al., 1995; Elliott et al., 1993; Geisbert et al., 1991; Kudoyarova-Zubavichene et al., 1999; Borisevich et al., 1995, 1996) and monoclonal murine IgG (Ikegami et al., 2003; Kazachinskaia et al., 2000; Ksiazek et al., 1992; Lucht et al., 2003; Niikura et al., 2001; Shahhosseini et al., 2007; Wilson et al., 2000). Smaller, recombinant single chain antibody (scFv) fragments comprised of only the variable domains of an IgG antibody linked by a flexible linker have also been derived both from immune tissues of an infected cynomolgus macaque (Druar et al., 2005) and from the naïve human scFv library "Griffin-1" (Tikunova et al., 2005; Nissim et al., 1994).

Traditional mono- and polyclonal antibody reagents as well as scFv fragments require provision of refrigerated conditions to maintain viability. However, immunodiagnostic reagents developed for in-field detection and diagnostics of tropical diseases such as Ebolavirus haemorrhagic fever ideally would be thermostable in these remote environments. Camelids and sharks are known to possess functional homodimeric antibodies composed of only heavy chains in addition to classical heterodimeric immunoglobulin antibodies (De Genst et al., 2006; Dooley and Flajnik, 2006). The variable domains of these antibodies, V_HH in camelids (Hamers-Casterman et al., 1993) and IgNAR V in sharks (Greenberg et al., 1995) have been shown to be highly thermostable (Dumoulin et al., 2002; Dooley et al., 2003). These single domain antibodies (sdAbs), therefore offer substantial potential for the generation of new immuno-detection reagents for the development of assays with improved characteristics for in-field use. As with the variable domains of other immunoglobulin scaffolds, camelid V_HH and shark IgNAR V domains are comprised of four highly conserved regions termed framework regions (FR) and three regions of higher variability in both number and composition of amino acids termed Complementary Determining Regions (CDRs). In comparison to other immunoglobulin domains the IgNAR V variable regions contain an unusually small CDR2 (Greenberg et al., 1995), which for structural reasons has recently been termed hypervariable region (HV) 2 (Dooley et al., 2006). In both camelids and sharks the CDR3 domain is much longer than that seen in other antibody domains. The CDR3 loop is stabilised by the presence of an inter-loop disulphide residues between CDR1 and CDR3 in camels and in type II IgNAR V domains in sharks or to the CDR2 loop in llamas (Roux et al., 1998; Harmsen and De Haard, 2007). In type I IgNAR V domains, extra cysteine residues are present in the framework (FR) 2 and 4 regions with additional pairs of cysteines in CDR3. These additional cysteine groups are thought to form either intra-CDR3 stabilising thiol bonds (Roux et al., 1998) or bonds between the CDR3 cysteine residues and those observed in the FR2 and FR4 regions to provide stabilisation (Diaz et al., 2002). Small size and increased internal disulphide bonding are likely to be the cause of the ability of camelid and shark domains to resist irreversible denaturation (Dooley et al., 2003; Perez et al., 2001).

A general advantage to the use of recombinant antibodies such as scFv and sdAb is they are easily combined with *in vitro* selection processes such as phage display to provide a rapid screening method for antibodies of desirable characteristics (Wesolowski et al., 2009). Genes encoding populations of immunoglobulin proteins can be readily generated from naïve animals (Sherwood et al., 2007; Liu et al., 2007a) and be further manipulated at the genetic level to increase diversity (Liu et al., 2007b; Shao et al., 2007). Alternatively libraries can be generated from tissues of animals immunised with desired antigens (Dooley et al., 2003, 2006). Whilst antibodies recently identified from a naïve camelid antibody library have shown excellent sensitivity and broad cross reactivity to antigens of interest including Marburgvirus (Sherwood et al., 2007), in general antibodies isolated from naïve and semi-synthetic shark derived scaffold libraries have shown affinities substantially lower than those achieved with conventional monoclonal reagents (Liu et al., 2007a,b). Immunisation of sharks has however resulted in the isolation of high affinity antibodies to protein targets from relatively small display libraries (Dooley et al., 2003, 2006). In order to generate robust reagents for the development of immunological assays towards *Ebolavirus* species the immunisation approach was used to improve the probability of generating high quality reagents. Previous reports have shown that sharks are able to raise immune responses to particulate agents (Clem and De Boutard, 1967; Suran et al., 1967; Frommel et al., 1971). In this study, we report the first targeted generation of a specific IgNAR V response to a complex particulate antigen in the nurse shark. More conventional Ebolavirus-specific recombinant reagents (scFv) were also generated using the same immunogen in a murine system. Furthermore, thermostability of IgNAR V was then directly compared to both scFv and murine monoclonal antibody (mAb), providing an indication of the benefits of utilising an IgNAR V in immunodiagnostic assays for use in challenging environmental conditions.

2. Materials and methods

2.1. Generation of immune response to Ebolavirus in sharks and mice

One male (shark 1) and one female (shark 2) Ebolavirus naïve nurse sharks (*Ginglymostoma cirratum*), each approximately 1.2 m in length, were procured and housed in the National Aquarium Holding Facility in Baltimore, USA. Each shark was immunised with 250 μg gamma irradiated inactivated sucrose gradient purified whole *Zaire ebolavirus* (USAMRIID) according to previously published methods (Dooley et al., 2003). Test bleed serum was taken from the tail sinus after each immunisation and assayed by Western Blot and ELISA for *Zaire ebolavirus* reactive IgNAR antibodies. A single female BALB/c mouse received a primary immunisation of the same immunogen in the presence of Freund's Complete Adjuvant (FCA), was boosted after three weeks and subsequently every 2 weeks until a satisfactory anti-Ebolavirus IgG-specific titre $\leq$1/10k was generated. The mouse then received pre-fusion boosts without adjuvant at least 10 weeks after the last boost with adjuvant.

2.2. Construction of scFv and IgNAR V phage display libraries

RNA was isolated from peripheral blood lymphocytes (PBLs) of immune sharks or mouse spleen respectively. Spleen tissue was ground in liquid nitrogen to generate a fine powder prior to processing. Both spleen tissue and PBLs were then lysed using TRIzol® Reagent (Gibco®). Total RNA was extracted from the homogenates via chloroform extraction followed by isopropanol precipitation. To generate IgNAR V gene cDNA fragments, 1 μg of RNA was added to Ready-To-Go™ 2-step RT-PCR Beads (Amersham Biosciences) using primers NARF4For1, NARF4For2, and NARF1Rev (Dooley et al., 2003). The RT-PCR product was purified and digested using *Nco* I and *Not* I and ligated into a similarly digested pHENII phage display vector. For the murine scFv library, first strand cDNA was amplified from total RNA using a SuperscriptII™ RT-PCR kit (Invitrogen). Gene fragments encoding the variable heavy (V_H) and variable light (V_L) antibody domains were then amplified using standard protocols using mixed primer sets to ensure library diversity (Burmester and Pluckthun, 2001). Overlap extension PCR was performed using primers scFor and scBack to generate intact scFv

fragments (Krebber et al., 1997). The scFv products were purified then digested using primer encoded *Sfi* I sites and cloned into the phage display vector pAK100 (Krebber et al., 1997).

Ligated plasmids from the IgNAR V pHENII and scFv pAK100 libraries were electroporated into competent XL-1 blue *E. coli* cells and plated onto 2xYT media containing 1% (w/v) glucose and either 100 μg ml^{-1} ampicillin or 30 μg ml^{-1} chloramphenicol, respectively. After overnight incubation (37 °C) colonies from each library were then collected from the plates and stored at −80 °C glycerol (50% (v/v) glycerol) stocks prior to use.

2.3. Affinity selection of IgNAR V and scFv libraries against Ebolavirus antigen

Phage carrying IgNAR V or scFv were generated by infection of bacterial stocks carrying phagemid plasmids (pHENII or pAK100) with M13K07 helper phage according to standard protocols (Krebber et al., 1997; Dooley et al., 2003). Affinity selection was then performed using sandwich-assay style process incorporating a negative selection step to remove any clones reactive to either Vero cell proteins present in the immunogen or the rabbit polyclonal antibody used to capture the antigen during the selection process.

Rabbit anti-Zaire IgG polyclonal antibody was immobilised overnight on Star Immunotubes (MaxiSorp™ StarTubes, Nunc). One immunotube was used as the negative selection tube and was coated with Vero cell lysate (University of Marburg) prior to addition of blocking buffer (2% (w/v) skimmed milk powder in PBS). The second tube was blocked prior to addition of inactivated *Zaire ebolavirus* (5 μg ml^{-1} in blocking buffer). One ml of phage preparation (1×10^{11} phage in blocking buffer with addition of 0.1% (v/v) Tween® 20) was added to the negative selection tube, which was incubated, rotating at room temperature for 1 h. The content of the negative panning tube was then transferred to the positive panning tube for a 20 min incubation rotating at room temperature. The positive panning tube was vigorously washed 20 times with PBS 0.1% (v/v) Tween 20™ and then 20 times with PBS, to remove non-binding phage. Bound phage were then eluted using 1 ml 100 mM triethylamine and neutralised with 1 M Tris–HCl pH 7.5. The neutralised phage were allowed to infect *E. coli* XL-1 Blue cells and the number of output phage were titred by serial dilution of a sample of this culture. Remaining cells were plated onto 2xYT media supplemented with 1% glucose and either 100 μg ml^{-1} ampicillin or 30 μg ml^{-1} chloramphenicol for the pHENII and pAK100 respectively and incubated at 37 °C overnight. Bacterial growth was harvested and a glycerol stock was prepared by resuspending the bacterial pellet in 50% (v/v) glycerol in 2xYT media, to be used in further rounds of selection.

2.4. Expression of recombinant antibodies in bacterial cell culture

Constructs for the expression of recombinant IgNAR V and scFv were generated using pAK300 vectors (Krebber et al., 1997) as fusion proteins with a murine kappa light chain constant domain. Single-chain antibody fragments were subcloned from the pAK100 phage display vector using the compatible *Sfi* I sites in each vector. IgNAR V gene fragments were amplified from the pHENII vector system using gene-specific primers containing compatible restriction sites. Expression media (2xYT, 0.1% (w/v) glucose containing 30 μg ml^{-1} chloramphenicol) was inoculated with bacterial glycerol stock to an Optical Density at 600 nm (OD_{600}) of 0.05 and incubated shaking (180 rpm) at 26 °C until the OD_{600} reached 0.5–0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM and the incubation continued for 16–24 h. Cells were then harvested from the cultures by centrifugation. Active recombinant protein was then precipitated from the supernatant by addition of ammonium sulphate (60% saturation) then dialysed against 40 mM Tris–HCl, 750 mM NaCl pH 7.5. Recombinant protein carrying a histidine tag was purified by nickel ion affinity purification using a 1 ml HisTrap column (Amersham Biosciences) that was connected to an Akta™ FPLC system (Amersham Biosciences). Elution fractions corresponding to eluted proteins were collected and dialysed (one million-fold) against PBS.

2.5. Western blot of Ebolavirus preparations

Samples of reduced and denatured, inactivated, purified *Zaire ebolavirus* or recombinant Ebolavirus proteins (USAMRIID) were added to 4× lithium dodecyl sulphate (LDS) sample buffer (Invitrogen™) and 10% (v/v) 10× reducing agent (Invitrogen™) then electrophoresed on 4–12% NuPAGE® bis-tris gels (Invitrogen™) using 3-(N-morpholino)propanesulphonic acid sodium dodecyl sulphate (MOPS SDS) running buffer (Invitrogen™). Ten μl of marker mix [50% SeeBlue™ Plus 2, 50% MagicMark™ XP, (Invitrogen™)] was added to control wells. Proteins were transferred onto polyvinylidene fluoride (PVDF) membrane using NuPAGE® bis-tris gel reducing transfer buffer (Invitrogen™). Blots were blocked in 5% (w/v) skimmed milk powder, 0.1% (v/v) Tween® 20 in PBS then probed using sera, recombinant IgNAR V or scFv. Bound antibody was detected using anti-Immunoglobulin New Antigen Receptor (IgNAR) pool comprising four anti-IgNAR-specific murine monoclonal antibodies (University of Maryland) or anti-mouse-HRP (Sigma) for shark or mouse antibodies respectively. Anti-IgNAR V was subsequently detected using anti-mouse-HRP (Sigma). The blots were then developed using ECL detection reagent (Amersham Biosciences) and visualised by exposing light sensitive film (Amersham).

2.6. Determination of specificity of antibody preparations using ELISA

Sera reactivity of immunised animals was determined using ELISA against directly immobilised *Zaire ebolavirus.* Serum samples were serially diluted and then the presence of bound antibody was detected by either goat anti-mouse IgG HRP (Sigma) (murine sera) or anti-NAR pool followed by goat anti-mouse IgG HRP (shark serum). For analysis of the reactivity of phage clones 10^9 monoclonal or polyclonal phage preparations were added to duplicate wells representing positive (*Zaire ebolavirus*) and negative (Vero cell preparation or rabbit IgG) antigens. Bound phage was detected using monoclonal anti-M13-HRP conjugate (Amersham). Sandwich ELISA was used to evaluate combinations of antibodies, as capture and detection reagents, for the ability to report the specific binding of filoviruses. To determine characteristics as capture reagents, purified recombinant scFv or IgNAR V was added to the ELISA plate. A dilution series of sample antigen (virus or negative control preparation) was then added. Rabbit polyclonal anti-Ebolavirus purified IgG was added and subsequently detected using goat anti-rabbit IgG-HRP conjugate (Biorad). The characteristics of the recombinant reagents were also determined using the antibodies in the opposite orientation (i.e. polyclonal anti-rabbit as the capture reagent).

2.7. Assessment of antibody resistance to irreversible thermal denaturation

IgNAR V, scFv and IgG proteins were incubated in solution at elevated temperatures for 3 h. Any remaining activity of the antibodies was then tested to determine the point at which these antibody classes were no longer able to function. This experiment closely followed previously published protocols (Dooley et al., 2003). Two existing monoclonal antibodies (DSTL027 and DSTL006), two scFv antibodies (DSTL094 and DSTL095) and two

IgNAR V antibodies (DSTL096 and DSTL097) were used in this experiment. Each antibody was initially titrated against inactivated *Zaire ebolavirus* at a dilution of 1:1000 in carbonate–bicarbonate buffer. A sub-saturating concentration, approximately 80–90% of the plateau value, was then selected for each antibody. The aliquots of each antibody were then incubated for 3 h in a Mastercycler® gradient (Eppendorf) thermal cycler. A control aliquot of antibody was maintained at room temperature (approximately 20 °C). At the end of the incubation, samples were allowed to cool to room temperature before analysis. An indirect ELISA was performed on a 96-well microtitre plate on which inactivated Ebolavirus had been immobilised. Incubation at each temperature was performed on three occasions and the mean for each data set was calculated and the antigen-negative background was subtracted. The percentage of the room temperature control signal was calculated for each antibody at each temperature.

3. Results

3.1. Generation of immune response in nurse shark (G. cirratum)

Test bleed serum taken from immunised sharks was assayed by Western blot and ELISA for IgNAR *Zaire ebolavirus* reactivity following each of the first three immunisations. An IgNAR response to *Zaire ebolavirus* was evident by ELISA (Fig. 1A,C) and Western Blot (Fig. 1B,D) following the second immunisation in both sharks. The ELISA endpoint titre of the shark serum did not increase between immunisations 2 and 3 (Fig. 1, A,C (bleed 3)). Western blot analysis did however demonstrate the benefit of continued immunisation by indicating both an increase in band intensity relating to NP and the broadening of response to include VP40 between bleeds 2 and 3 (Fig. 1B,D, lanes marked 3 and 4). Serum component fractionation by Sephacryl™ S-300 gel filtration chromatography also suggested that the immune responses of the two sharks were maturing, as the fractions containing proteins of molecular weights similar to IgNAR and monomeric IgM increased in absorbance (280 nm) over the immunisation time course (results not shown). The positive response observed to virus in ELISA and the progression of immune response observed by Western blot indicated that a *Zaire ebolavirus* reactive IgNAR serum fraction had successfully been generated.

3.2. Screening of IgNAR V libraries for specific binders to Ebolavirus

Two libraries of 2.6×10^7 and 2.5×10^7 members (defined as an individual cell line in a library prior to amplification through growth) were generated from shark 1 and shark 2 respectively. Two rounds of bio-panning were performed with each library to enrich for antibodies with specificity to *Zaire ebolavirus*. Analysis of the binding activity of polyclonal populations of phage from each library after each round of selection demonstrated that enrichment of phage with specificity to *Zaire ebolavirus* was successful (Fig. 2). Binding attributable to milk proteins, rabbit IgG and Vero cell proteins remained at background levels following the two rounds of bio-panning. The binders derived from the library generated from shark 1 were greater in number than those for shark 2 however, the increase in shark 2 binders were deemed sufficient to warrant screening of individual clones to identify phage displaying IgNAR V of interest.

Twenty-four monoclonal phage preparations from each library were screened by sandwich ELISA to identify *Zaire ebolavirus* reactive clones. Twenty-one (88%) of the clones from shark 1 were positive (as determined by an absorbance greater than double the background value) by ELISA, compared to seven (29%) positive clones from shark 2. The positive clones were ranked according to absorbance, to enable two clones (Shark 1: derived DSTL096 and Shark 2: derived DSTL097) to be selected for further evaluation.

Sequences of DSTL096 and DSTL097 confirm that these IgNAR V were both type 1 IgNAR genes (Fig. 2B). The framework regions of both sequences were fully conserved in relation to published germline sequence (Dooley et al., 2003). Both genes were also highly conserved within CDR1 with only two variations between sequences both of which involve synonymous amino acids. Amino acid 71 located within CDR/HV 2 changed with potential significance from serine (hydroxyl side chain) in DSTL096 to tryptophan (aromatic side chain) in DSTL097. In IgNAR V domains CDR3 is the main determinant of antigen interaction (Diaz et al., 1998). Within

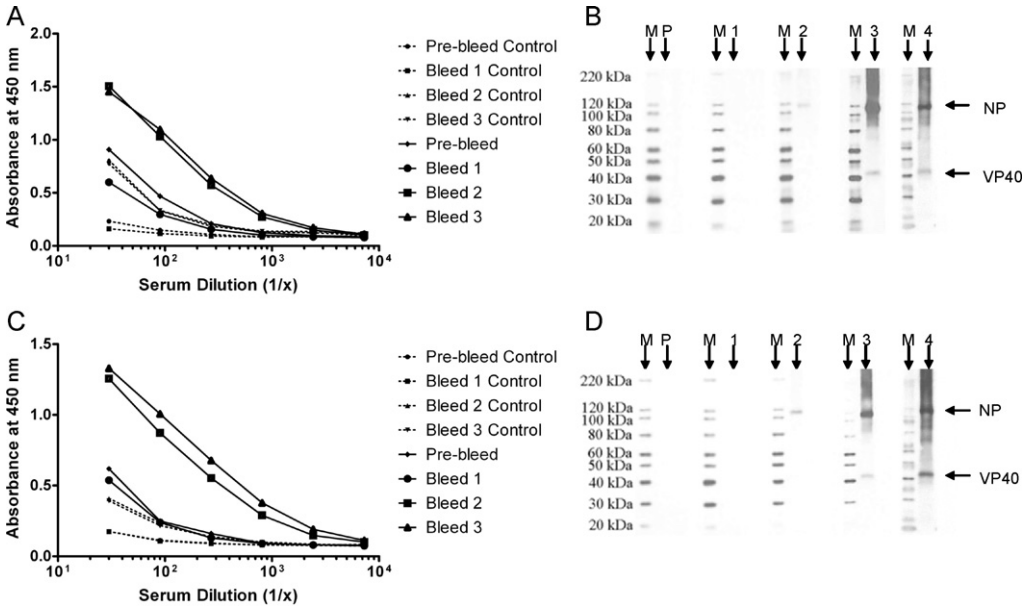

Fig. 1. Generation of immune response to *Zaire ebolavirus* in sharks. ELISA analysis of serum for the development of IgNAR V response to virus is shown in (A) and (C) for shark 1 and shark 2 respectively. Western blot analysis of pre-bleed serum (1) and serum collected following the 1st (2), 2nd (3), 3rd (4) and 4th (5) immunisations highlighting the development of response to VP40 and NP viral proteins are shown for both shark 1 (B) and shark 2 (D) respectively.

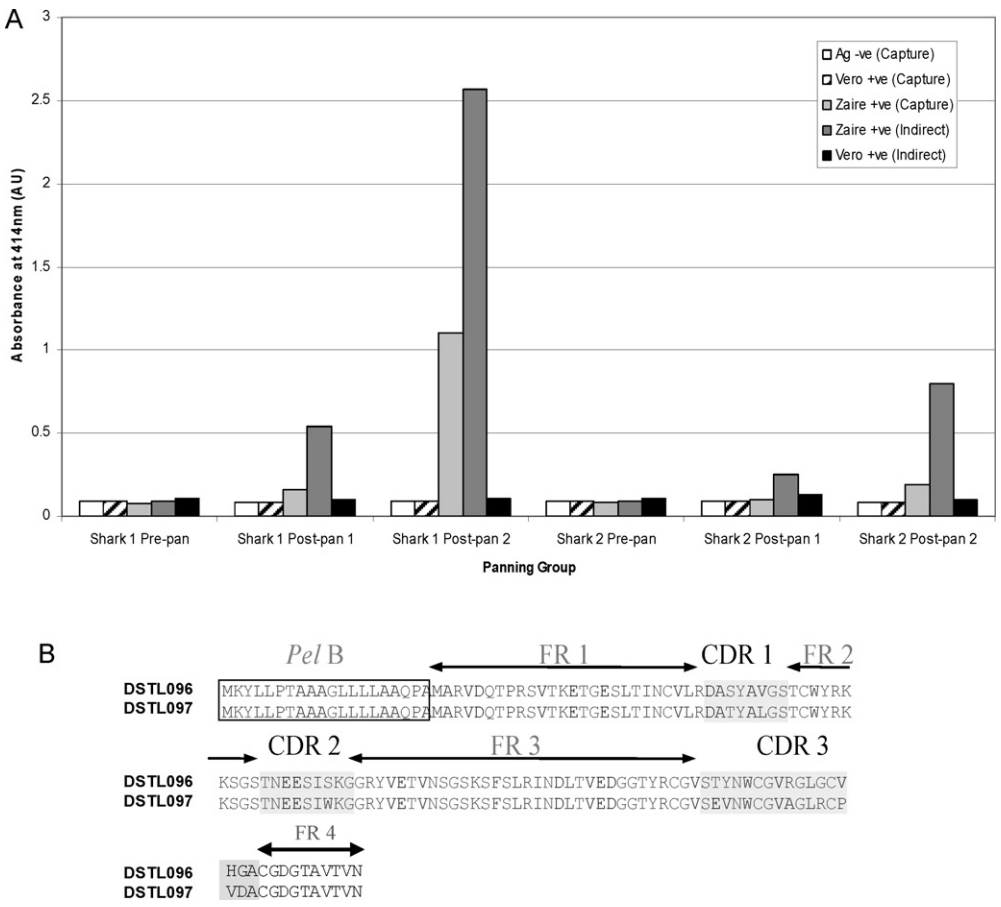

Fig. 2. Isolation of IgNAR V antibody domains with specificity to Ebolavirus. (A) Two rounds of affinity selection were performed for each IgNAR V phage display against capture immobilised *Zaire ebolavirus* leading to specific enrichment of the libraries for clones with specificity to the viral antigen. (B) Sequences of DSTL096 and DSTL097 generated from the affinity selection of the shark 1 and shark 2 libraries respectively.

this region six amino acid differences exist between the CDR3 of DSTL096 and DSTL097 in addition to one synonymous substitution.

3.3. Screening of murine scFv library for the isolation of binders to Zaire ebolavirus

A library of 2.4×10^7 members was generated from a single immunised mouse and subjected to two rounds of bio-panning incorporating a negative selection step in the same manner as the IgNAR V libraries. Progress towards enrichment of the library for Ebolavirus-specific clones was monitored by analysis of the binding reactivity of the phage populations towards immobilised antigen in ELISA. After two rounds of bio-panning it was demonstrated that in contrast to the IgNAR V bio-panning, no enrichment for *Zaire ebolavirus* reactivity was achieved. The library was however enriched for the ability to bind rabbit IgG, despite the inclusion of this antibody in the negative selections step (results not shown). The murine scFv library was therefore panned against Ebolavirus antigen immobilised directly on the immunotube surface. Two rounds of bio-panning were conducted; at each round, exposure to immobilised rabbit IgG and Vero cell proteins was performed (negative selection) prior to positive selection on immobilised virus. This strategy was successful in generating pools of phage that showed a robust binding signal towards *Zaire ebolavirus* (Fig. 3). A high background binding response was however observed to Vero cell preparation (Fig. 3). Thirty-two single clones from the second round of bio-panning were assessed for binding to inactivated *Zaire ebolavirus*, Vero cell preparation, rabbit IgG and blocked plastic. None of the 32 clones showed reactivity to rabbit IgG or blocked plastic. Surprisingly given the polyclonal phage reactivity, none of the phage stocks showed reactivity to Vero cell preparation. Twenty six (81%) of the phage stocks demonstrated reactivity to *Zaire ebolavirus* presented in both indirect and sandwich ELISA formats. Amongst positive phage stocks, all showed a higher absorbance to directly immobilised virus compared to antibody captured virus (results not shown). The positive clones were ranked by absorbance values and the best eight clones were submitted for sequencing. Based on this analysis two unique clones (Fig. 3B) were selected for further evaluation (DSTL094, DSTL095).

The sequence analysis of the CDRs of DSTL094 and DSTL095 does not appear substantially different with only three amino acid differences: heavy chain CDR1 arginine for lysine and heavy chain CDR2 glycine for serine and histidine for tyrosine. Although several changes occur in the FR, it is unlikely that these changes significantly impact on antigen interaction. The lengths of the poly-glycine (GGGGS) linker peptide differ between the two antibodies as a result of the variability of the overlap-extension PCR reaction used to generate the scFv gene fragments. It remains unclear what impact this has on the stability of scFv.

3.4. Analysis of the binding specificity of IgNAR V and scFv

IgNAR V and scFv isolated by the bio-panning process were produced as soluble proteins to allow further characterisation of their binding properties in the absence of the phage scaffold. Western blot analysis against viral proteins, host cell proteins and baculovirus-expressed recombinant (r) *Zaire ebolavirus* proteins demonstrated that both DSTL096 and DSTL097 reacted to the viral

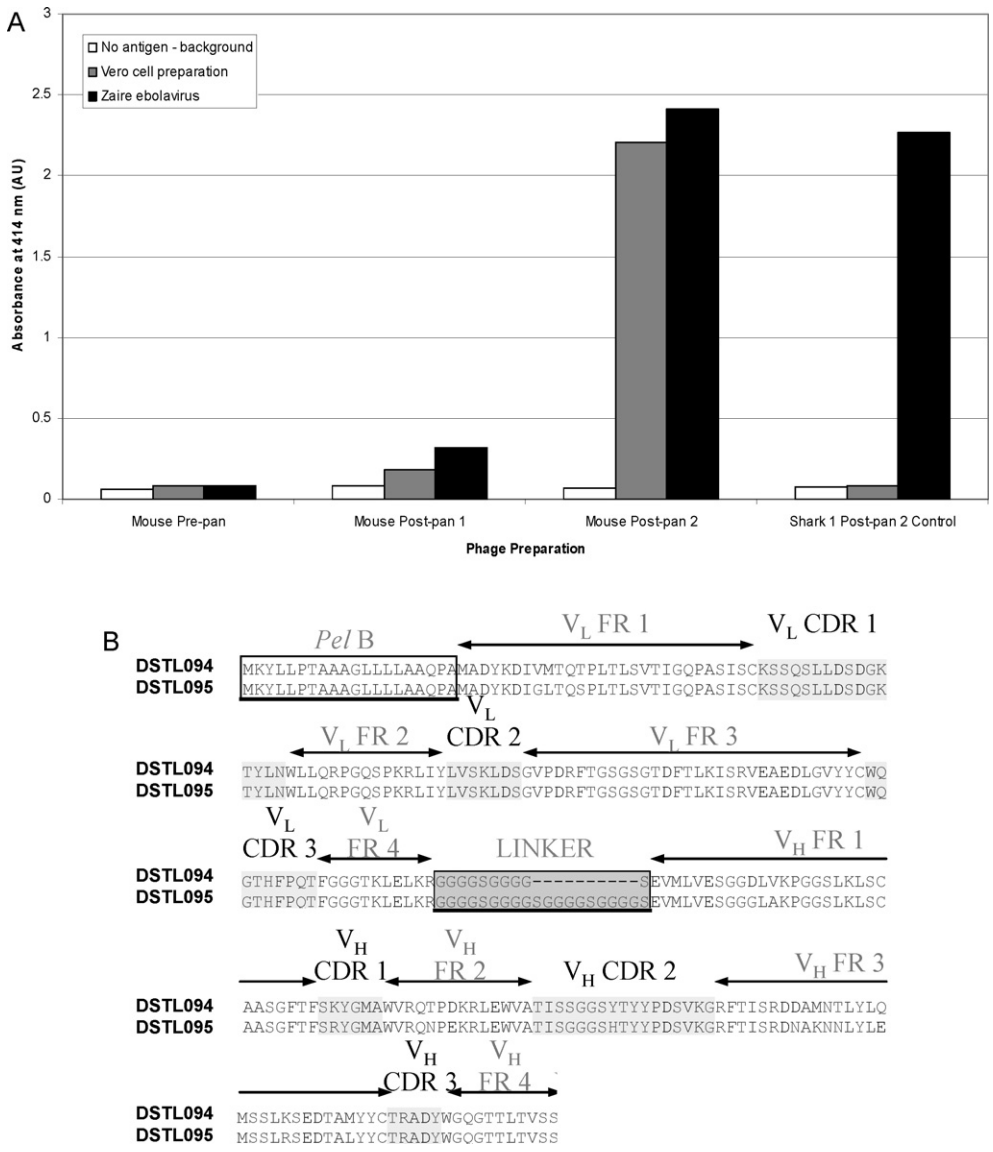

Fig. 3. Isolation of scFv with specificity to *Zaire ebolavirus*. (A) ELISA results from polyclonal populations of phage after each round of bio-panning show enrichment of reactivity of the library to immobilised *Zaire ebolavirus* and Vero cell lysate. (B) Sequences of DSTL094 and DSTL095 scFv isolated from the library.

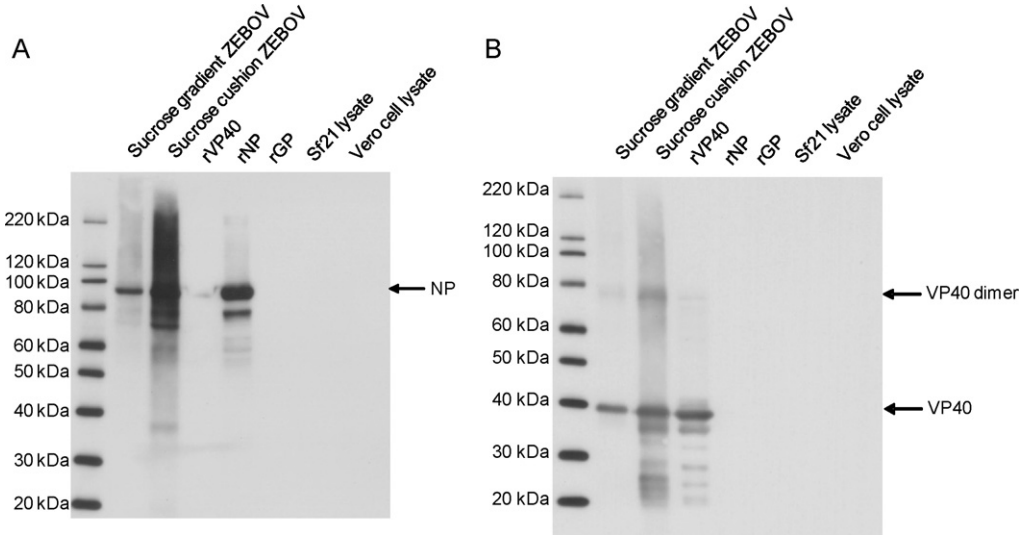

Fig. 4. Western blot analysis of the binding specificity of recombinant (A) IgNAR V DSTL096 and (B) scFv DSTL095 scFv to whole purified *Zaire ebolavirus* (ZEBOV) and recombinant forms of *Zaire ebolavirus* protein components.

nucleoprotein (Fig. 4A). No reactivity was observed to rVP40, rGP, migrated Vero cell or Sf21 cell lysates. In contrast to the reactivity observed for IgNAR Vs, two scFvs (DSTL094 and DSTL095) recognised VP40 (Fig. 4B). The isolation and characterisation of additional recombinant antibodies from both mouse and shark may add insight into whether the difference in target specificity observed with these four antibodies is indicative of a difference in immunodominance of viral proteins in the two animal systems. Monitoring of the development of the murine immune response by Western blot, in a manner similar to that described earlier for the shark system, may also provide important information on this topic.

Recombinant antibodies were tested for their ability to capture *Zaire ebolavirus* in sandwich ELISA. The ability of each antibody to capture a range of *Ebolavirus* species was tested in a directly comparable ELISA using live agent in BSL-4 using the same dilution series prepared independently in a 96-tube array. All antibodies performed well as capture reagents. An assay using any of the IgNAR V reagents as a capture reagent was able to detect all the *Ebolavirus* species, except *Côte d'Ivoire ebolavirus*, whilst maintaining no reactivity to *Lake Victoria marburgvirus* and mock infected Vero cell preparation (Fig. 5). In contrast, assays using either of the scFv reagents as the capture antibody were only able to detect *Zaire ebolavirus* (Fig. 6). The comparative limits of detection for the assays using scFv were higher than the IgNAR V utilising assays. It is possible that this was as a result of freeze-thaw damage enhanced by the presence of imidazole in the scFv preparations, which had been removed by dialysis from the IgNAR V preparations. The response curve pattern from assays involving DSTL097 and DSTL096 appeared sufficiently different to suggest the targeting of a different epitope or a significant difference in complementarity for the same epitope. This was apparent in the improvement in the limit of detection for *Reston ebolavirus* [6×10^4 pfu ml^{-1} (DSTL096) to 2×10^3 pfu ml^{-1} (DSTL097)] and *Sudan ebolavirus* [2×10^5 pfu ml^{-1} (DSTL96) to 7×10^3 pfu ml^{-1} (DSTL97)]. In comparison to a polyclonal reagent, they performed less well as detection reagents (results not shown). This was expected given that there was likely to be far fewer epitopes available to a monoclonal reagent than a polyclonal reagent. No combination showed reactivity to a mock infected Vero cell preparation.

3.5. Comparative assessment of the thermal stability of IgNAR V, scFv and monoclonal antibody

The ability of IgNAR V (DSTL096 and DSTL097) and scFv (DSTL094 and DSTL095) to withstand irreversible thermal denaturation from short (3 h) incubations at elevated temperatures was investigated in comparison to existent murine monoclonal antibody reagents (DSTL006, DSTL027). The percentage activity in comparison to duplicate samples held at room temperature for the 3 h period was calculated and the mean values from triplicate experiments were plotted (Fig. 7). Significant differences between the three types of recognition element were apparent. The two scFv representatives were fully inactivated by the 3 h incubation at 50 °C. The scFv DSTL095 was substantially more thermolabile than DSTL094, with DSTL094 and DSTL095 retaining 95% and 6% activities, respectively, following incubation at 40 °C. The low activity retained by DSTL095 was unexpected given that this temperature is so close to the temperature used for ELISA incubation stages (37 °C). Inactivation of the two mAb representatives was uniform with both mAbs retaining almost complete activity following incubation at 60 °C (91 ± 4% and 94 ± 2% for DSTL006 and DSTL027, respectively) and complete activity loss following incubation at 70 °C. The large standard deviation from the mean value observed at the lower incubation temperatures for DSTL006 suggests that the values in excess of 100% are within the experimental error. Inactivation of DSTL096

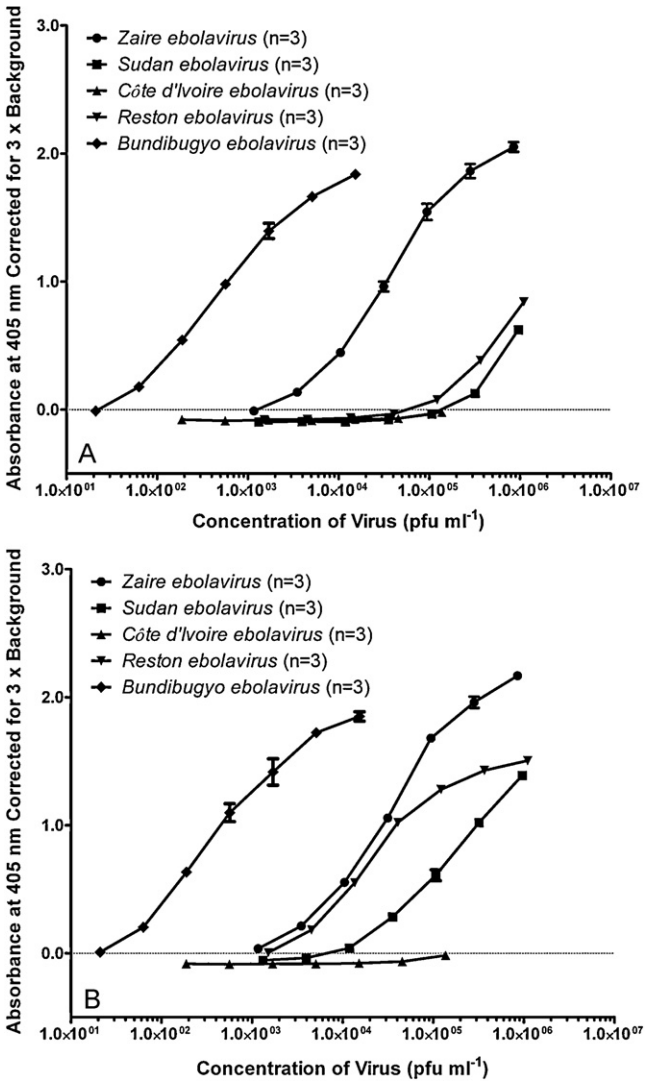

Fig. 5. Reactivity to *Ebolavirus* species in ELISA using (A) DSTL096 and (B) DSTL097. Antigen dilution series were constructed with viruses represented in duplicate and mock antigens (either Vero cell or CV-7 cell preparation) included without replication. Rabbit anti-Zaire diluted to 10 μg ml^{-1} was used as the detection reagent. The antigen-negative background mean (n = 12) plus three standard deviations was subtracted from all data points.

and DSTL097 begins at the same point (60 °C) as the mAb representatives, however in contrast, approximately 80% activity and up to 42 ± 1% (DSTL097) activity (DSTL096, 31 ± 3% activity) were observed for the IgNAR V representatives at 70 °C and 80 °C respectively. Full inactivation was observed following 3 h incubation at 90 °C. The prolonged tolerance followed by complete inactivation observed with both of the mAb representatives might suggest an inability to re-fold following thermal denaturation. The longer shoulder over which IgNAR V was capable of retaining partial activity might be due to an ability to re-fold following thermal denaturation. Therefore, the superior performance of IgNAR V over the other two types of immunoglobulin evaluated in this study is likely to be due to tolerance to irreversible thermal denaturation as opposed to being resistant to thermal denaturation. At elevated temperatures of incubation where activity was not detectable, samples containing scFv and mAb produced a white precipitate; such precipitate was not observed in the IgNAR V samples. The white precipitate was considered to be insoluble protein although this was not confirmed. This observation would support the suggestion that it is the resistance to irreversible denaturation that is important. Such an ability

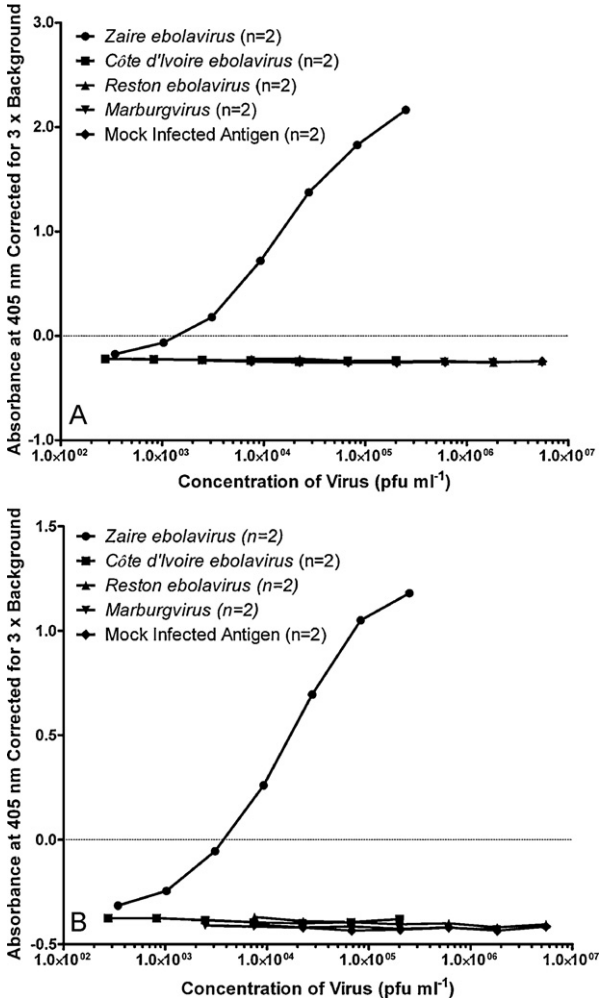

Fig. 6. Activity of murine scFv as capture reagents for *Ebolavirus* species. (A) DSTL094 and DSTL095 were used as capture reagents in ELISA using anti-Zaire rabbit polyclonal as the detection reagent.

to re-fold into an active conformation is likely to stem from the small domain size and the high level of internal disulphide bonding. The exposed areas of hydrophobicity are likely to be equally important from a negative perspective for the poor performance of scFv. It was never considered likely that a large multi-domain tetramer would re-fold correctly and this was borne out in this experiment.

4. Discussion

This work describes the successful development of recombinant murine scFv and shark IgNAR V antibodies specific to Ebolavirus. Both scFv and IgNAR V antibodies were generated from the tissues of animals immunised with *Zaire ebolavirus* and for sharks this represents the first example of a targeted IgNAR V isolated as a result of immune response generated as a result of immunisation to a particulate antigen.

Mice immunised with *Zaire ebolavirus* were used to generate two recombinant scFv that were specific for the viral matrix protein (VP40). Further to this murine monoclonal antibodies generated to Ebolavirus have also shown a propensity for binding to VP40 (unpublished data). This provides evidence to suggest that VP40 may be the immunodominant protein for IgG reactivity in mice. Comparatively, in two different sharks, the serological time course analysis of serum by Western blot demonstrated that a response to the viral nucleoprotein preceded a response to VP40. Both of the IgNAR V isolated in this work were shown to have specificity for NP. This evidence may indicate that NP may be the immunodominant protein for IgNAR binding in the nurse shark. Although, a greater body of evidence would be needed for this difference in immune recognition to be verified, if correct, this could be exploited when developing biological recognition elements to other particulate antigens.

The nucleoprotein (NP) is the first of the viral structural proteins to be produced in an infected cell and constitutes 17% of the total virion protein (Elliott et al., 1993). Immunodominant epitopes are located within the carboxyl-terminal 110 amino acids (Saijo et al., 2001). Whilst epitopes within NP have been identified by immunoassay that may be of use in discriminating Ebolavirus isolates (Niikura et al., 2001), the high sequence similarity between the protein sequences of NP within the *Ebolavirus* genus indicate that it is also an excellent target for the generation of cross reactive antibodies. This theory has been borne out in this work, illustrated by the breadth of Ebolavirus cross reactivity shown by the IgNAR V domains, in particular DSTL097. The NP protein forms part of the central ribonucleoprotein complex of the virus particle within the host derived viral envelope. The reactivity of NP-specific IgNAR V to live virus could therefore be considered to be surprising. Previous reports however describe the "moth-eaten" appearance of Ebolavirus particles under electron microscopy, suggesting that the viral envelope is disrupted in places across the virion (Geisbert and Jahrling, 1995; Ellis et al., 1979). It is therefore likely that the ribonucleoprotein complex is available for antibody binding through the disrupted Ebolavirus envelope.

VP40 represents a more traditional target for the generation of detection and diagnostic assays due to the prevalence of this protein within the virion particle (37.7%) (Elliott et al., 1993). Antibodies with reactivity to VP40 such as the scFv materials generated in this work, which function as compatible capture and detection reagents in sandwich ELISA, have recently been described as both necessary and unattained for diagnostic testing (Shahhosseini et al., 2007). Although in this work the sensitivity of assays using the IgNAR V domains specific to NP and scFv specific to VP40 are comparable, assays that target VP40 rather than any other viral protein are likely to be more sensitive, when sensitivity is measured in virions, which may be of importance when looking for the earliest stages of viraemia. The scFv generated within this work may therefore offer some advantage in generating a highly sensitive assay for diagnostic use. Western blot analysis of sera from immunised sharks indicates some reactivity to VP40. Therefore, the availability of a large recombinant IgNAR V library does offer the potential for the isolation of additional VP40-specific reagents if this is required for the development of future assays. However, whilst the ability of scFv generated in this study to capture only *Zaire ebolavirus* provides utility in a panel of assays by providing discrimination between those species considered most hazardous to man, as an initial identification reagent, the IgNAR V ability to capture all species of *Ebolavirus* implicated in major human disease outbreaks may be of greater immediate utility.

All of the recombinant antibodies were shown to capture viable virus in a sandwich ELISA. This characteristic is important where the capture of an antigen onto a solid phase is the basis of many biosensor assay formats. However, the assays using both scFv and IgNAR V described in this work are less sensitive for the detection of *Reston ebolavirus* than for the other *Ebolavirus* species tested. Since *Zaire ebolavirus* was used to generate the immune response in the animal models a reduction in sensitivity for *Reston ebolavirus* species is not surprising. Whilst human EVHF as a result of infection with *Reston ebolavirus* has not been reported, seroconversion has been identified, suggesting sub-clinical viral replication. In addition, *Reston ebolavirus* is seeming pathogenic for certain non-human primates

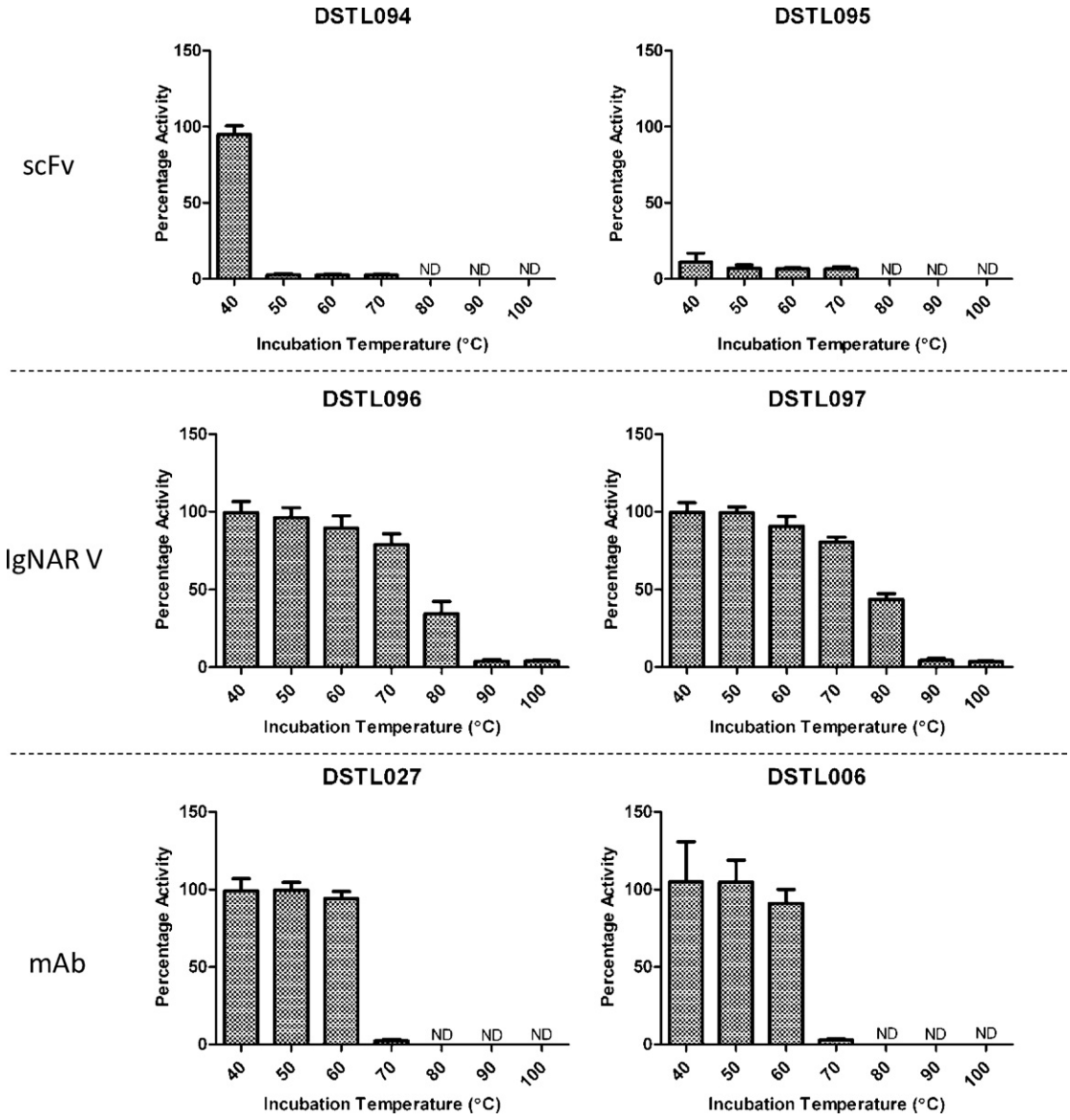

Fig. 7. The effect on activity following incubation of IgNAR V, scFv and mAb for 3 h at a range of temperatures. The absorbance generated in indirect ELISA against inactivated *Zaire ebolavirus* from sub-saturating concentrations of IgNAR V, scFv or mAb that had been held for 3 h at a range of temperatures between 40 °C and 100 °C were compared to duplicate control samples held at room temperature. Mean values were calculated from the triplicate data points available in each of the three experiments ($n = 9$). Error bars indicate 95% confidence limits; ND, not determined. Mean absorbance values for each antibody (abs 414 nm) at sub-saturating concentration were as follows: DSTL094, 2.43 ± 0.06 ($n = 36$); DSTL095, 0.985 ± 0.03 ($n = 36$); DSTL096, 1.43 ± 0.04 ($n = 64$); DSTL097, 1.46 ± 0.04 ($n = 64$); DSTL027, 2.51 ± 0.11 ($n = 36$); DSTL006, 2.07 ± 0.11 ($n = 36$). ± = 95% Confidence Limits.

as *Zaire ebolavirus* is for humans (Sanchez et al., 2001). Frequent human exposure to such a highly mutable RNA virus carries a significant risk of generating a strain of *Reston ebolavirus* capable of causing haemorrhagic fever in humans. Therefore, further work to identify either scFv or IgNAR V to more broadly cover this species could be envisioned.

Titres of Ebolavirus in infected subjects have been shown to reach high levels in non-human primate models with viraemias recorded at peaks of $1 \times 10^{6.9}$ pfu ml^{-1} and $1 \times 10^{5.47}$ pfu ml^{-1} on day 6 of infection in examples of infection in cynomolgus macaques and rhesus macaques respectively (Geisbert et al., 2003; Hensley et al., 2007). Similarly high titres of virus have also been recorded in human cases (WHO, 1978) and in both non-human primate and human cases of infection, a viraemia lower than $1 \times 10^{4.5}$ pfu ml^{-1} is strongly associated with survival (Sanchez et al., 2007). The application of the IgNAR V and scFv molecules derived in this study to clinically relevant materials has not been determined. However, the detection limits derived for *Zaire ebolavirus* using the scFv isolated in this work are below these levels. For the IgNAR V, DSTL096 provides promising detection limits for both *Zaire ebolavirus* and *Bundibugyo ebolavirus* with IgNAR V DSTL097 showing greatest coverage of species including *Zaire ebolavirus*, *Bundibuygo ebolavirus*, *Reston ebolavirus* and *Sudan ebolavirus*. This provides encouragement that these reagents could offer some utility in a diagnostic application if the function of the reagents is maintained in a clinically relevant sample type.

As expected, IgNAR V was shown to offer increased thermostability over both conventional murine IgG and murine scFv. Previous studies investigating the stability of other murine scFv show that this antibody format is unstable in heat treatment studies with one example having a half life of 15 h at 37 °C (Glockshuber et al., 1990) and a further example of an scFv produced as a fusion protein with human κ constant domain showed only 34% residual activity after incubation for 3 h at 41 °C (Dooley et al., 1998). In comparison to

these previous studies, scFv DSTL094 performs well, retaining 95% activity after incubation for 3 h at 40 °C. The amino acid sequences for scFv did not appear uncharacteristic of murine scFv, so it is probable that this highly labile behaviour is typical of murine scFv.

The recombinant binding domain of camelid IgG is also described as thermostable (Dumoulin et al., 2002), although the published assessments are not directly comparable to the data available for IgNAR V (Dooley et al., 2003; Ladenson et al., 2006). It would appear that the thermostability observed both here and by Dooley et al. (2003) with IgNAR V reagents, is unlikely to be linked to the small size of the variable domain alone, but more as a result of other factors such as fewer exposed hydrophobic patches and increased intra-molecular disulphide bonding. It was unfortunate that a type II IgNAR V was not generated, as the impact on thermostability of the variation in putative disulphide bond formation could not be investigated.

The thermostability and breadth of specificity of the IgNAR V reagents generated in this work is encouraging. Distinct advantages in terms of reagent stability have been demonstrated, above the characteristics of IgG and scFv, highlighting the potential of shark derived IgNAR V reagents for the development of versatile assays with stabilities that would enable use in areas of disease outbreak without the need for an associated cold storage facility (Grolla et al., 2005). For the first time the ability to generate specific immune responses in a shark model also offers a robust route for the generation of high quality IgNAR V to agents of interest as required, meeting this aspiration.

Acknowledgements

The authors gratefully acknowledge the input of Dr. Martin Pearce, Dr. Carl Mayers and Dr. Clive Sweet for useful discussion and technical advice within this work, as well as Nicola Walker, Brian Kearney, Matthew Voorhees, Tamara Clements, Scott Olschner and Aubrey Harbaugh for valuable assistance. Additionally, the authors gratefully acknowledge funding for this work by the Dstl Innovation Fund and the Dstl Programme Office on behalf of the UK Ministry of Defence.

References

Alibek, K., 2000. Biohazard. Arrow, London.

Borio, L., Inglesby, T., Peters, C.J., Schmaljohn, A.L., Hughes, J.M., Jahrling, P.B., Ksiazek, T., Johnson, K.M., Meyerhoff, A., O'Toole, T., Ascher, M.S., Bartlett, J., Breman, J.G., Eitzen Jr., E.M., Hamburg, M., Hauer, J., Henderson, D.A., Johnson, R.T., Kwik, G., Layton, M., Lillibridge, S., Nabel, G.J., Osterholm, M.T., Perl, T.M., Russell, P., Tonat, K., 2002. Hemorrhagic fever viruses as biological weapons: medical and public health management. JAMA 287, 2391–2405.

Borisevich, I.V., Mikhailov, V.V., Krasnianskii, V.P., Gradoboev, V.N., Lebedinskaia, E.V., Potryvaeva, N.V., Timan'kova, G.D., 1995. Development and study of the properties of immunoglobulin against Ebola fever. Vopr. Virusol. 40, 270–273.

Borisevich, I.V., Mikhailov, V.V., Potryvaeva, N.V., Malinkin Iu, N., Kirillov, A.P., Krasnianskii, V.P., Markov, V.I., Makhlai, A.A., Lebedinskaia, E.V., 1996. [Development of the immunoenzyme test-system for detection of Ebola virus antigen]. Vopr. Virusol. 41, 232–234.

Broussard, L.A., 2001. Biological agents: weapons of warfare and bioterrorism. Mol. Diagn. 6, 323–333.

Burmester, J., Pluckthun, A., 2001. Construction of scFv fragments from hybridoma or spleen cells by PCR assembly. In: Dubel, S. (Ed.), Antibody Engineering. , first ed. Springer, Heidelberg, pp. 19–40.

CDC, 2000. Biological and chemical terrorism: strategic plan for preparedness and response. Recommendations of the CDC Strategic Planning Workgroup. MMWR Recomm. Rep. 49, 1–14.

Chepurnov, A.A., Merzlikin, N.V., Chepurnova, T.S., Vorob'eva, M.S., 1994. Preparation of rabbit antiserum to Ebola virus. Vopr. Virusol. 39, 286–288.

Clem, L.W., De Boutard, F., 1967. Studies on elasmobranch immunoglobulins. In: Gilbert, P.W., Mathewson, R.F., Rall, D.P. (Eds.), Sharks, Skates, and Rays. The Johns Hopkins Press, Baltimore, MD, pp. 581–591.

De Genst, E., Saerens, D., Muyldermans, S., Conrath, K., 2006. Antibody repertoire development in camelids. Dev. Comp. Immunol. 30, 187–198.

Diaz, M., Greenberg, A.S., Flajnik, M.F., 1998. Somatic hypermutation of the new antigen receptor gene (NAR) in the nurse shark does not generate the repertoire: possible role in antigen-driven reactions in the absence of germinal centers. Proc. Natl. Acad. Sci. U.S.A. 95, 14343–14348.

Diaz, M., Stanfield, R.L., Greenberg, A.S., Flajnik, M.F., 2002. Structural analysis, selection, and ontogeny of the shark new antigen receptor (IgNAR): identification of a new locus preferentially expressed in early development. Immunogenetics 54, 501–512.

Dooley, H., Flajnik, M., 2006. Antibody repertoire development in cartilaginous fish. Dev. Comp. Immunol. 30, 43–56.

Dooley, H., Flajnik, M.F., Porter, A.J., 2003. Selection and characterization of naturally occurring single-domain (IgNAR) antibody fragments from immunized sharks by phage display. Mol. Immunol. 40, 25–33.

Dooley, H., Grant, S.D., Harris, W.J., Porter, A.J., 1998. Stabilization of antibody fragments in adverse environments. Biotechnol. Appl. Biochem. 28, 77–83.

Dooley, H., Stanfield, R.L., Brady, R.A., Flajnik, M.F., 2006. First molecular and biochemical analysis of in vivo affinity maturation in an ectothermic vertebrate. Proc. Natl. Acad. Sci. U.S.A. 103, 1846–1851.

Druar, C., Saini, S.S., Cossitt, M.A., Yu, F., Qiu, X., Geisbert, T.W., Jones, S., Jahrling, P.B., Stewart, D.I., Wiersma, E.J., 2005. Analysis of the expressed heavy chain variable-region genes of *Macaca fascicularis* and isolation of monoclonal antibodies specific for the Ebola virus' soluble glycoprotein. Immunogenetics 57, 730–738.

Dumoulin, M., Conrath, K., Van Meirhaeghe, A., Meersman, F., Heremans, K., Frenken, L.G., Muyldermans, S., Wyns, L., Matagne, A., 2002. Single-domain antibody fragments with high conformational stability. Protein Sci. 11, 500–515.

Elliott, L.H., Sanchez, A., Holloway, B.P., Kiley, M.P., McCormick, J.B., 1993. Ebola protein analyses for the determination of genetic organization. Arch. Virol. 133, 423–436.

Ellis, D.S., Stamford, S., Lloyd, G., Bowen, E.T.W., Platt, G.S., Way, H., Simpson, D.I.H., 1979. Ebola and Marburg viruses. I. Some ultrastructural differences between strains when grown in vero cells. J. Med. Virol. 4, 201–211.

Frommel, D., Litman, G.W., Finstad, J., Good, R.A., 1971. The evolution of the immune response. XI. The immunoglobulins of the horned shark, *Heterodontus francisci*: purification, characterisation and structural requirement for antibody activity. J. Immunol. 106, 1234–1243.

Geisbert, T.W., Hensley, L.E., Larsen, T., Young, H.A., Reed, D.S., Geisbert, J.B., Scott, D.P., Kagan, E., Jahrling, P.B., Davis, K.J., 2003. Pathogenesis of Ebola hemorrhagic fever in cynomolgus macaques. Am. J. Pathol. 163, 2347–2370.

Geisbert, T.W., Jahrling, P.B., 1995. Differentiation of Filoviruses by electronmicroscopy. Virus Res. 39, 129–150.

Geisbert, T.W., Rhoderick, J.B., Jahrling, P.B., 1991. Rapid identification of Ebola virus and related filoviruses in fluid specimens using indirect immunoelectron microscopy. J. Clin. Pathol. 44, 521–522.

Georges-Courbot, M.C., Lu, C.Y., Lansoud-Soukate, J., Leroy, E., Baize, S., 1997. Isolation and partial molecular characterisation of a strain of Ebola virus during a recent epidemic of viral haemorrhagic fever in Gabon. Lancet 349, 181.

Glockshuber, R., Malia, M., Pfitzinger, I., Pluckthun, A., 1990. A comparison of strategies to stabilize immunoglobulin Fv-fragments. Biochemistry 29, 1362–1367.

Greenberg, A.S., Avila, D., Hughes, M., Hughes, A., McKinney, E.C., Flajnik, M.F., 1995. A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. Nature 374, 168–173.

Groen, J., van den Hoogen, B.G., Burghoorn-Maas, C.P., Fooks, A.R., Burton, J., Clegg, C.J., Zeller, H., Osterhaus, A.D., 2003. Serological reactivity of baculovirus-expressed Ebola virus VP35 and nucleoproteins. Microbes Infect. 5, 379–385.

Grolla, A., Lucht, A., Dick, D., Strong, J.E., Feldmann, H., 2005. Laboratory diagnosis of Ebola and Marburg hemorrhagic fever. Bull. Soc. Pathol. Exot. 98, 205–209.

Hamers-Casterman, C., Atarhouch, T., Muyldermans, S., Robinson, G., Hamers, C., Songa, E.B., Bendahman, N., Hamers, R., 1993. Naturally-occurring antibodies devoid of light chains. Nature 363, 446–448.

Harmsen, M.M., De Haard, H.J., 2007. Properties, production and applications of camelid single-domain antibody fragments. Appl. Microbiol. Biotechnol. 77, 13–22.

Hensley, L.E., Stevens, E.L., Yan, B., Geisbert, J.B., Macias, W.L., Larsen, T., Daddario-DiCaprio, K.M., Cassell, G.H., Jahrling, P.B., Geisbert, T.W., 2007. Recombinant human activated protein C for the postexposure treatment of Ebola hemorrhagic fever. JID 196, S390–S399.

ICTV, 2005. Virus Taxonomy – 2005. ICTV website, based on the 8th report of the ICTV edn: International Committee on Taxonomy of Viruses. www.ictvonline.org.

Ikegami, T., Niikura, M., Saijo, M., Miranda, M.E., Calaor, A.B., Hernandez, M., Acosta, L.P., Manalo, D.L., Kurane, I., Yoshikawa, Y., Morikawa, S., 2003. Antigen capture enzyme-linked immunosorbent assay for specific detection of *Reston ebolavirus* nucleoprotein. Clin. Diagn. Lab. Immunol. 10, 552–557.

Kazachinskaia, E.I., Pereboev, A.V., Chepurnov, A.A., Belanov, E.F., Razumov, I.A., 2000. Monoclonal antibodies to Ebola virus: isolation, characteristics, and study of cross reactivity with Marburg virus. Vopr. Virusol. 45, 40–44.

Khan, A.S., Tshioko, F.K., Heymann, D.L., Le Guenno, B., Nabeth, P., Kerstiens, B., Fleerackers, Y., Kilmarx, P.H., Rodier, G.R., Nkuku, O., Rollin, P.E., Sanchez, A., Zaki, S.R., Swanepoel, R., Tomori, O., Nichol, S.T., Peters, C.J., Muyembe-Tamfum, J.J., Ksiazek, T.G., 1999. The reemergence of Ebola hemorrhagic fever, Democratic Republic of the Congo, 1995. Commission de Lutte contre les Epidemies a Kikwit. J. Infect. Dis. 179 (Suppl. 1), S76–86.

Krebber, A., Bornhauser, S., Burmester, J., Honegger, A., Willuda, J., Bosshard, H.R., Pluckthun, A., 1997. Reliable cloning of functional antibody variable domains from hybridomas and spleen cell repertoires employing a reengineered phage display system. J. Immunol. Methods 201, 35–55.

Ksiazek, T.G., Rollin, P.E., Williams, A.J., Bressler, D.S., Martin, M.L., Swanepoel, R., Burt, F.J., Leman, P.A., Khan, A.S., Rowe, A.K., Mukunu, R., Sanchez, A., Peters, C.J.,

1999a. Clinical virology of Ebola hemorrhagic fever (EHF): virus, virus antigen, and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic of the Congo, 1995. J. Infect. Dis. 179 (Suppl. 1), S177–187.

Ksiazek, T.G., West, C.P., Rollin, P.E., Jahrling, P.B., Peters, C.J., 1999b. ELISA for the detection of antibodies to Ebola viruses. J. Infect. Dis. 179 (Suppl. 1), S192–198.

Ksiazek, T.G., Rollin, P.E., Jahrling, P.B., Johnson, E., Dalgard, D.W., Peters, C.J., 1992. Enzyme immunosorbent assay for Ebola virus antigens in tissues of infected primates. J. Clin. Microbiol. 30, 947–950.

Kudoyarova-Zubavichene, N.M., Sergeyev, N.N., Chepurnov, A.A., Netesov, S.V., 1999. Preparation and use of hyperimmune serum for prophylaxis and therapy of Ebola virus infections. J. Infect. Dis. 179 (Suppl. 1), S218–S223.

Ladenson, R.C., Crimmins, D.L., Landt, Y., Ladenson, J.H., 2006. Isolation and characterisation of a thermally stable recombinant anti-caffine heavy chain antibody fragment. Anal. Chem. 78, 4501–4508.

Leroy, E.M., Baize, S., Lu, C.Y., McCormick, J.B., Georges, A.J., Georges-Courbot, M.C., Lansoud-Soukate, J., Fisher-Hoch, S.P., 2000. Diagnosis of Ebola haemorrhagic fever by RT-PCR in an epidemic setting. J. Med. Virol. 60, 463–467.

Liu, J.L., Anderson, G.P., Delehanty, J.B., Baumann, R., Hayhurst, A., Goldman, E.R., 2007a. Selection of cholera toxin specific IgNAR V single-domain antibodies from a naïve shark library. Mol. Immunol. 44, 1775–1783.

Liu, J.L., Anderson, G.P., Goldman, E.R., 2007b. Isolation of anti-toxin single domain antibodies from a semi-synthetic spiny dogfish shark display library. BMC Biotechnol. 7, 78.

Lloyd, E.S., Zaki, S.R., Rollin, P.E., Tshioko, K., Bwaka, M.A., Ksiazek, T.G., Calain, P., Shieh, W.J., Konde, M.K., Verchueren, E., Perry, H.N., Manguindula, L., Kabwau, J., Ndambi, R., Peters, C.J., 1999. Long-term disease surveillance in Bandundu region, Democratic Republic of the Congo: a model for early detection and prevention of Ebola hemorrhagic fever. J. Infect. Dis. 179 (Suppl. 1), S274–S280.

Lucht, A., Grunow, R., Moller, P., Feldmann, H., Becker, S., 2003. Development, characterization and use of monoclonal VP40-antibodies for the detection of Ebola virus. J. Virol. Methods 111, 21–28.

Merzlikin, N.V., Chepurnov, A.A., Istomina, N.N., Ofitserov, V.I., Vorob'eva, M.S., 1995. Development and application of an immunoenzyme test system for diagnosing Ebola fever. Vopr. Virusol. 40, 31–35.

Niikura, M., Ikegami, T., Saijo, M., Kurane, I., Miranda, M.E., Morikawa, S., 2001. Detection of ebola viral antigen by enzyme-linked immunosorbent assay using a novel monoclonal antibody to nucleoprotein. J. Clin. Microbiol. 39, 3267–3271.

Nissim, A., Hoogenboom, H.R., Tomlinson, I.M., Flynn, G., Midgley, C., Lane, D., Winter, G., 1994. Antibody fragments from a "single pot" phage display library as immunological reagents. EMBO J. 13, 692–698.

Perez, J.M., Renisio, J.G., Prompers, J.J., van Platerink, C.J., Cambillau, C., Darbon, H., Frenken, L.G., 2001. Thermal unfolding of a llama antibody fragment: a two-state reversible process. Biochemistry 40, 74–83.

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

Rodriguez, L.L., De Roo, A., Guimard, Y., Trappier, S.G., Sanchez, A., Bressler, D., Williams, A.J., Rowe, A.K., Bertolli, J., Khan, A.S., Ksiazek, T.G., Peters, C.J., Nichol, S.T., 1999. Persistence and genetic stability of Ebola virus during the outbreak in Kikwit, Democratic Republic of the Congo, 1995. J. Infect. Dis. 179 (Suppl. 1), S170–S176.

Roux, K.H., Greenberg, A.S., Greene, L., Strelets, L., Avila, D., McKinney, E.C., Flajnik, M.F., 1998. Structural analysis of the nurse shark (new) antigen receptor (NAR): molecular convergence of NAR and unusual mammalian immunoglobulins. Proc. Natl. Acad. Sci. U.S.A. 95, 11804–11809.

Rowe, A.K., Bertolli, J., Khan, A.S., Mukunu, R., Muyembe-Tamfum, J.J., Bressler, D., Williams, A.J., Peters, C.J., Rodriguez, L., Feldmann, H., Nichol, S.T., Rollin, P.E., Ksiazek, T.G., 1999. Clinical, virologic, and immunologic follow-up of convalescent Ebola hemorrhagic fever patients and their household contacts, Kikwit, Democratic Republic of the Congo. Commission de Lutte contre les Epidemies a Kikwit. J. Infect. Dis 179 (Suppl. 1), S28–S35.

Saijo, M., Niikura, M., Morikawa, S., Ksiazek, T.G., Meyer, R.F., Peters, C.J., Kurane, I., 2001. Enzyme-linked immunosorbent assays for detection of antibodies to Ebola and Marburg viruses using recombinant nucleoproteins. J. Clin. Microbiol. 39, 1–7.

Sanchez, A., Geisbert, T.W., Feldmann, H., 2007. Filoviridae: Marburg and Ebola viruses. In: Knipe, D., Howley, P. (Eds.), Fields Virology. , fifth ed. Lippincott Williams and Wilkins, Philadelphia, pp. 1409–1448.

Sanchez, A., Khan, A.S., Zaki, S.R., Nabel, G.J., Ksiazek, T.G., Peters, C.J., 2001. Filoviridae: Marburg and Ebola viruses. In: Howley, P.M. (Ed.), Fields Virology. , fourth ed. Lippincott Williams & Wilkins, Philadelphia.

Shahhosseini, S., Das, D., Qiu, X., Feldmann, H., Jones, S.M., Suresh, M.R., 2007. Production and characterization of monoclonal antibodies against different epitopes of Ebola virus antigens. J. Virol. Methods 143, 29–37.

Shao, C.-Y., Secombes, C.J., Porter, A.J., 2007. Rapid isolation of IgNAR variable single-domain antibody fragments from a shark synthetic library. Mol. Immunol. 44, 656–665.

Sherwood, L.J., Osborn, L.E., Carrion, R., Patterson, J.L., Hayhurst, A., 2007. Rapid assembly of sensitive antigen-capture assays for Marburg virus, using and in vitro selection of llama single-domain antibodies at biosafety level 4. JID 196, S213–219.

Suran, A.A., Tarail, M., Papermaster, B.W., 1967. Immunoglobulins of the Leopard shark: I. Isolation and characterisation of 17S and 7S immunoglobulins with precipitating activity. J. Immunol. 99, 679–686.

Tikunova, N.V., Batanova, T.A., Chepurnov, A.A., 2005. Human recombinant antibodies to Ebola virus: preparation and characteristics. Vopr. Virusol. 50, 25–29.

Towner, J.S., Sealy, T.K., Khristova, M.L., Albarino, C.G., Conlan, S., Reeder, S.A., Quan, P.-L., Lipkin, W.I., Downing, R., Tappero, J.W., Okware, S., Lutwama, J., Bakamutumaho, B., Kayiwa, J., Comer, J.A., Rollin, P.E., Ksiazek, T.G., Nichol, S.T., 2008. Newly discovered Ebola virus associated with hemorrhagic fever outbreak in Uganda. PLoS Pathogens 4, 1–6.

Towner, J.S., Rollin, P.E., Bausch, D.G., Sanchez, A., Crary, S.M., Vincent, M., Lee, W.F., Spiropoulou, C.F., Ksiazek, T.G., Lukwiya, M., Kaducu, F., Downing, R., Nichol, S.T., 2004. Rapid diagnosis of Ebola hemorrhagic fever by reverse transcription-PCR in an outbreak setting and assessment of patient viral load as a predictor of outcome. J. Virol. 78, 4330–4341.

Wesolowski, J., Alzogaray, V., Reyelt, J., Unger, M., Juarez, K., Urrutia, M., Cauerhff, A., Danquah, W., Rissiek, B., Scheuplein, F., Schwarz, N., Adriouch, S., Boyer, O., Seman, M., Licea, A., Serreze, D.V., Goldbaum, F.A., Haag, F., Kock-Nolte, F., 2009. Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. Med. Microbiol. Immunol. 198, 157–174.

WHO, 1978. Ebola haemorrhagic fever in Zaire 1976 – Report of an International Commission. Bulletin of the World Health Organisation 56, 271–293.

Wilson, J.A., Hevey, M., Bakken, R., Guest, S., Bray, M., Schmaljohn, A.L., Hart, M.K., 2000. Epitopes involved in antibody-mediated protection from Ebola virus. Science 287, 1664–1666.