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Development of humanised antibodies for Crimean-Congo Haemorrhagic fever virus: Comparison of hybridoma-based versus phage library techniques

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ABSTRACT

Humanised antibodies targeting Crimean-Congo Haemorrhagic virus (CCHFV) are needed for the development and standardisation of serological assays. These assays are needed to address a shortfall in available tests that meet regulatory diagnostic standards and to aid surveillance activities to extend knowledge on the distribution of CCHFV. To generate a humanised monoclonal antibody against CCHFV, we have compared two methods: the traditional mouse hybridoma approach with subsequent sequencing and humanisation of antibodies versus a non-animal alternative using a human combinatorial antibody library (HuCAL). Our results demonstrated that the mouse hybridoma followed by humanisation protocol gave higher affinity antibodies. Whilst not yet able to demonstrate the generation of equivalent humanised antibodies without the use of animals, sequencing data enables the subsequent production of recombinant antibodies, thus providing a reduction in future animal usage for this application. Ultimately, our report provides information on development of a humanised standardised control, which can form an important positive control component of serological assays against CCHFV.

1. Introduction

Crimean-Congo Haemorrhagic Fever virus (CCHFV) is a tick-borne pathogen belonging to the genus *Orthonairovirus* of the family *Nairoviridae* within the order *Bunyavirales* (Adams et al., 2017) that is responsible for the disease CCHF. The disease was first brought to modern medical attention in 1945, when Soviet military personnel and local inhabitants developed acute haemorrhagic fevers following humanitarian operations during and after the Crimean offensive. However, the aetiology of this disease was not recognised until 1967 when the virus was isolated. Interestingly, it was shown to be identical to Congo virus that had been isolated and characterised in 1958 at the East African Virus Research Institute in Uganda. The terminology 'Crimean-Congo' (Dowall et al., 2017), is a compromised, political name agreed during the cold war in the early 1970s. The presence of CCHFV continues to increase, both in endemic areas and in its expansion to new regions (Spengler et al., 2019). This spread is attributed to multiple factors such as climate change, increasing tick numbers, increasing exposure of animal and human hosts to ticks and advancement of detection assays (Belobo et al., 2021). With a global case fatality rate of 11.7% in humans with acute infection (Belobo et al., 2021) and due to its potential to cause major epidemics in humans, CCHFV is classified by the World Health Organisation (WHO) as a priority disease for research and development (Sweileh, 2017).

The status of CCHFV-specific antibodies in animal and human populations provide indicators for the presence or absence of the virus in specified areas. For development of assays, the nucleoprotein (NP) is widely chosen due to being antigenically conserved between strains (Liu et al., 2014; Moming et al., 2018), being strongly immunogenic (Dowall et al., 2016; Mazzola and Kelly-Cirino, 2019) and produced in high amounts early during infection. Whilst the CCHFV NP is also the most immunogenic antigen for humoral responses, it also elicits cellular immune responses during natural infection in humans (Karaaslan et al., 2021).

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Serological tests to detect antibodies to CCHFV NP have been widely developed, generally using different expression techniques to produce the recombinant protein including *E. coli* (Samudzi et al., 2012; Emmerich et al., 2018), baculovirus (Saijo et al., 2002; Dowall et al., 2012), Semliki Forest alphavirus (Garcia et al., 2006), plants (Atkinson et al., 2016) and mammalian cells (Lombe et al., 2021). Cross-reactivity has been shown for NP's covering the most diverse CCHFV strains e.g., from Greece (Aigai virus) and South Africa (SPU415/85) which have an 8.7% amino acid difference. It has also been demonstrated that recombinant NP prepared from geographical distinct strains retains diagnostic and epidemiological applications worldwide (Rangunwala et al., 2014).

As reported in 2012, we previously established an indirect ELISA method for the measurement of CCHFV IgG and IgM based on recombinant CCHFV NP (Dowall et al., 2012). This assay has been used for international serosurveillance studies including in Italy (De Liberato et al., 2018), Kosovo (Thomas et al., 2012) and Nigeria (Bukbuk et al., 2016). To further improve this assay, we have developed a standard positive control humanised antibody. In this study, a traditional technique of generating mouse hybridomas followed by 'humanising' with molecular grafting has been used and compared alongside a non-animal alternative approach using a human combinatorial antibody library (HuCAL) (Knappik et al., 2000; Krebs et al., 2001; Rothe et al., 2008; Prassler et al., 2011).

2. Materials and methods

2.1. Recombinant CCHFV-NP protein

The CCHFV-NP gene, containing an N-terminal His-tag, was inserted into *flash*BAC ULTRA™ DNA (Oxford Expression Technologies Ltd., Oxford, UK) under control of the baculovirus polyhedrin gene promoter to generate recombinant baculovirus (FBU-CCHFV-NP). Insect cells, 200 ml at 1.5×10^6 cells/ml (*Tricoplusia ni* Tn1, Oxford Expression Technologies Ltd., Oxford, UK), were infected with recombinant FBU-CCHFV-NP virus (at multiplicity of 5 plaque-forming units per cell) and cells were harvested at 72 h post-infection (King and Possee, 1992). The His-tagged CCHFV-NP protein (0.34 mg/ml) was purified by Cobalt-sepharose chromatography (Cytiva HiTrap™Talon™, Fisher, UK) and analysed by SDS PAGE.

2.2. Generation of hybridoma cell lines

Immunisations and hybridoma studies were conducted by Antibody Production Services, Bedford, UK. Four BALB/c mice were immunised three times over a period of five weeks with 1 mg/ml recombinant CCHFV NP. On day 45, serum samples were collected for assessment of antibody titre. The mice received an extra booster immunisation, following which an extra serum sample was collected for the assessment of antibody titre. Mice were culled and the spleens from the best two responding mice were fused with myeloma cells to generated hybridoma clones. Five hybridoma cell lines were used for cloning studies with supernatants assessed for stability of specific antibody production following which 2 were expanded as the final established hybridoma cell lines (EG5 and EA10).

2.3. Screening of mouse antibodies

High binding 96-well plates (Nunc Maxisorb; Thermo Fisher, Loughborough, UK) were coated with 1 μg/ml CCHFV NP dilution in carbonate-bicarbonate buffer overnight and blocked with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBS-Tween) and 0.2% gelatin for at least 1 h. Serial dilution concentrations of mouse sera or hybridoma supernatant were added for 1 h at room temperature. After washing with PBS containing 0.05% Tween-20, bound antibodies were detected using anti-mouse IgG coupled to horse-radish peroxidase (HRP) conjugate (Sigma, Poole, UK). Tetramethylbenzidine (TMB) substrate (Cheshire Scientific, Cheshire, UK) was added for 10–20 min followed by stop solution consisting of 1 M sulphuric acid. Absorbances were read at a wavelength of 450 nm.

2.4. Humanisation of mouse hybridomas

Humanisation of the mouse hybridoma cell line, EG5, was performed by Absolute Antibody, Cleveland, UK. The variable heavy and light chains (VH and VL, respectively) were sequenced. The complementarity-determining regions (CDR) were assigned using the international ImMunoGeneTics (IMGT) information system (Lefranc, 2003). Structure guided humanisation models were built using in-house software. VH and VL domains were designed to enable appropriate restriction sites at the 5′ and 3′ ends to enable cloning and codon optimised for expression in human cells. Following gene synthesis, the variable domains were cloned into Absolute Antibody vectors and sequence verified. Plasmid DNA for transfection were then generated for transfection in human embryonic kidney 293 (HEK293) cells. Cells were transiently transfected with the heavy and light chain expression vector and cultured for a further 6 days. Purification was performed by Protein A affinity chromatography with elution using citrate buffer, pH 3.0, followed by neutralisation with 0.5 M Tris, pH 9.0. The eluted protein was then exchanged into PBS. Antibody concentration was determined by UV spectroscopy. Antibody purity was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and high performance liquid chromatography (HPLC) using an appropriate size exclusion column (SEC).

2.5. Screening of humanised antibodies

Microtitre plates were coated with 5 μg/ml CCHFV NP for 1 h before blocking with PBS containing 1% casein for a further hour. Following washing with PBS-Tween, serial dilutions of antibody test samples was added in a 3-fold dilution series. After 1 h incubation, plates were washed with PBS-Tween and HRP labelled anti-human IgG antibody (Sigma, Poole, UK) before detection with TMB substrate (Fisher Scientific, Loughborough, UK). The reaction was stopped with 1 M hydrochloric acid and absorbances read at a wavelength of 450 nm.

2.6. HuCAL antibody generation

HuCAL protocols were performed by Bio-Rad AbD Serotec GmbH, Puchheim, Germany. The HuCAL PLATINUM phage library was panned for antibodies recognising CCHFV NP with 3 rounds of enrichment. The enriched antibody gene pool from the phage display vector were subcloned into a bacterial expression vector to generate antibodies in the Fab format. Following further screening against CCHFV NP, 20 clones were sequenced, expressed and purified. Three clones were converted into full-length human IgG1.

2.7. Screening of HuCAL antibodies

384-well plates (Maxisorp MTP; Thermo Scientific, Loughborough, UK) were coated with 5 μg/mL antigen. To each well, 20 μL HuCAL antibodies at a concentration of 2 μg/mL were added and left to bind for 1 h. Bound antibodies were detected using anti-FLAG-AP secondary antibody (Sigma, Poole, UK) and detected with AttoPhos reagent (Roche, Welwyn Garden City, UK). Excitation was measured at 400 nm with emission at 550 nm.

2.8. ELISA binding studies

To directly compare the fully humanised antibodies, an indirect CCHF ELISA was conducted as previously described (Dowall et al., 2012). Briefly, 96-well plates were coated with 2.5 μg/mL CCHFV NP. Antibodies were added to wells in triplicate, with a starting

concentration of 20 μg/mL and subsequent dilutions in a 5-fold dilution series. Bound antibodies were detected using a HRP-conjugated goat anti-human IgG Fc secondary antibody (Jackson ImmunoResearch, Ely, UK) following by addition of TMB substrate and acidic stop solution. Absorbances were read at a wavelength of 450 nm.

3. Results

3.1. Generation of murine hybridomas

Four mice were immunised with approximately 250 μg recombinant CCHFV NP antigen, three times over a period of five weeks. Sera samples were collected on day 45 to assess antibody levels. All mice demonstrated levels of antibodies specific to the antigen (Fig. 1a), but as the reciprocal serum dilution giving the half maximum absorbance reading was under 1:10,000, an extra immunisation was undertaken. The results from the second test bleed identified titres above the desired threshold (Fig. 2a) with mouse 1 and 2 showing the highest responses and being selected for hybridoma production.

Spleens cells from the two mice were fused with the myeloma cell line, the resulting hybridomas were seeded across ten 96-well plates per animal. Colonies were subsequently screened for the presence of CCHFV NP-specific antibodies and out of 1880 tested, five were taken forward for clonal amplification based on absorbance levels being above 0.5.

The five colonies were cloned through expansion and subsequent limiting dilution assays with ELISA confirmation of antibody secretion. Following the first round of cloning, two remained positive for stable antibody secretion that were further subcloned to generate clonal cell lines. Antibody from these cell lines were purified and standardised to a stock concentration of 1 mg/ml. Antibody titrations were analysed (Fig. 2) and the one with the highest level of binding, EG5, was taken forward for humanisation work.

3.2. Humanisation of murine hybridomas

The variable domains of the heavy (VH) and light (VL) regions of immunoglobulin from the EG5 hybridoma cell line were sequenced (Tables 1 and 2). After processing data through the ImMunoGeneTics (IMGT) Gap Align tool, the complementary determining regions (CDRs) aligned close to mouse sequences; specifically, the IGHV5–6-4 family for the VH and IGLV1 for the VL. Running the VH and VL data through a CDR grafting algorithm, transferred the murine sequences onto selected human germline sequences. Molecular models were built (Fig. 3) to enable structure guided humanisation and determine which of the framework amino acids to retain to maintain binding activity. Four humanised sequences were generated for each of the VH and VL regions

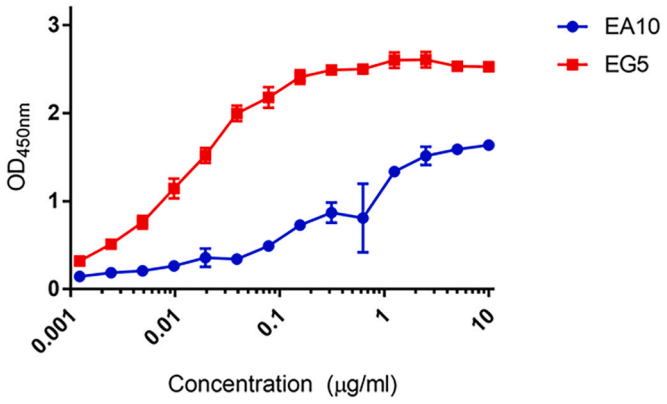

Fig. 2. Binding of two murine monoclonal antibody clones (EA10 red; EG5 blue) to recombinant CCHFV NP antigen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Tables 1 and 2). These were checked to ensure that they would be free of glycosylation and cysteines using a sequence liability tool.

The four humanised VH and VL chains were synthesised separately and cloned into human IgG1 heavy chain and human lambda light chain expression vectors, respectively. All possible combinations of the humanised sequences were made to create a total of 16 different humanised antibodies. The antibodies were expressed at small scale and the proteins purified by Protein A. All antibodies were successfully expressed.

3.3. Activity analysis of humanised antibodies

All 16 humanised antibodies were assessed for binding activity against recombinant CCHFV NP antigen. A range of activities were observed, with some showing strong binding potency, including the original chimeric antibody (Fig. 4a). The chimeric antibody and top five humanised antibodies were taken forward for further screening, with a lower starting concentration, to successfully achieve full titration (Fig. 4b). The IC50 values were calculated with four of the humanised antibodies (cAb4729–10.0, cAb4721–10.0, cAb4723–10.0 and cAb4731–10.0) showing activity within two-fold of the chimeric antibody (Table 3).

3.4. Generation of humanised antibodies through the HuCAL system

Recombinant CCHFV NP antigen was screened through the HuCAL

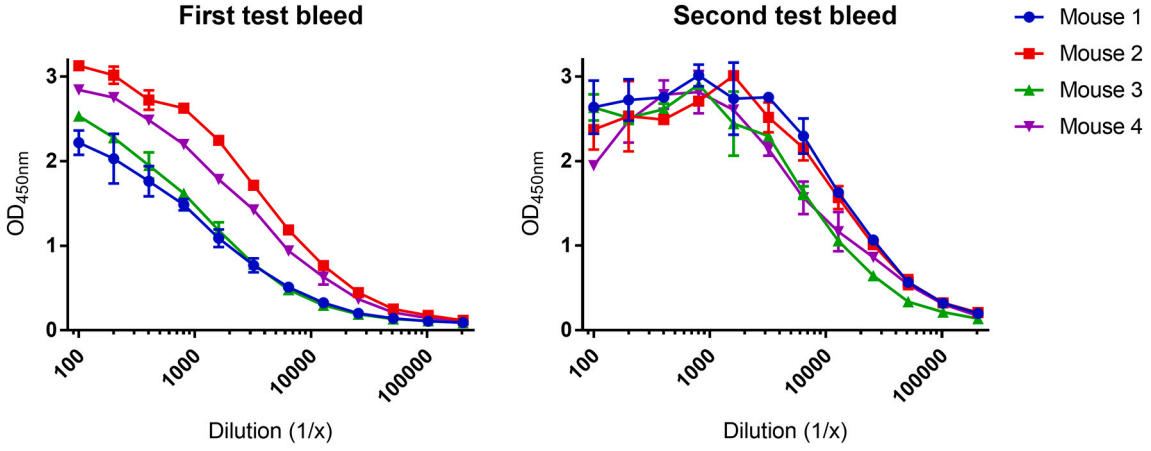

Fig. 1. Determination of antigen-specific antibody levels in mice immunised with recombinant CCHFV NP antigen. Responses after three (a) and four (b) immunisations are shown. Symbols show mean values with error bars denoting standard deviation.

Table 1
Sequence of the VH region from the murine EG5 hybridoma and generated humanised sequences.

Format	Human germline	Sequence	Identity to human
Mouse	–	DVKLVESGGGLVKPGGSLKLSCAASGFPFSSYTMSWLRQTPEKRLEWVATISSGGSYT QYPDSVKGRFTISRDNAKNTLYLQMSSLRSEDTAMYYCTRAPLFAYWGQGTLVTVSA	79.4%
Humanised	IGHV3–11*01	DVKLVESGGGLVKPGGSLRLSCAASGFPFSSYTMSWLRQAPGKGLEWVATISSGGSYT QYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCTRAPLFAYWGQGTLVTVSS	87.6%
Humanised	IGHV3–11*01	QVQLVESGGGLVKPGGSLRLSCAASGFPFSSYTMSWIRQAPGKGLEWVATISSGGSYT QYPDSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCTRAPLFAYWGQGTLVTVSS	89.8%
Humanised	IGHV3–72*01	DVKLVESGGGLVQPGGSLRLSCAASGFPFSSYTMSWLRQAPGKGLEWVATISSGGSYT QYPDSVKGRFTISRDNAKNSLYLQMNSLKTEDTAVYYCTRAPLFAYWGQGTLVTVSS	84.5%
Humanised	IGHV3–72*01	EVQLVESGGGLVQPGGSLRLSCAASGFPFSSYTMSWVRQAPGKGLEWVATISSGGSYT QYPDSVKGRFTISRDNSKNSLYLQMNSLKTEDTAVYYCTRAPLFAYWGQGTLVTVSS	85.7%

Table 2
Sequence of the VL region from the murine EG5 hybridoma and generated humanised sequences.

Format	Human germline	Sequence	Identity to human
Mouse	–	QAVVTQESALTTSPGETVTLTCRSSTGAVTTSNYANWVQEKPDHLFTGLIGGTNNRAPG VPARISGSLIGDKAALTITGAQTEDEAIYFCGLWYSNHLVFGGGTKLTVLG	62.1%
Humanised	IGLV7–4-6*01	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQQKPGQAFRGLIGGTNNRAPW TPARFSGSLIGDKAALTLSGAQPEDEAEYFCGLWYSNHLVFGGGTKLTVL	80.0%
Humanised	IGLV7–4-6*01	QAVVTQEPSLTVSPGGTVTLTCGSSTGAVTTSNYANWVQQKPGQAFRGLIGGTNNKASW TPARFSGSLLGDKAALTLSGAQPEDEAEYYCGLWYSNHLVFGGGTKLTVL	84.2%
Humanised	IGLV8–61*01	QAVVTQEPSFSVSPGGTVTLTCGSSTGAVTTSNYANWVQQTPGQAFRGLIGGTNNRAPG VPDRFSGSLIGDKAALTITGAQADDESDYFCGLWYSNHLVFGGGTKLTVL	74.0%
Humanised	IGLV8–61*01	QAVVTQEPSFSVSPGGTVTLTCGSSTGAVTTSNYANWVQQTPGQAFRGLIGGTNNRASG VPDRFSGSLLGDKAALTITGAQADDESDYYCGLWYSNHLVFGGGTKLTVL	77.1%

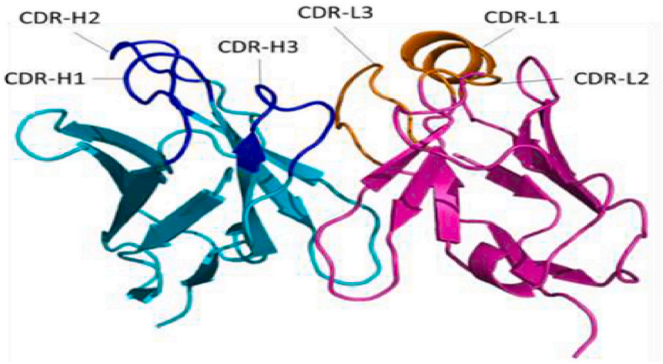

Fig. 3. Model of the EG5 VH and VL domains. The VH is coloured in cyan with dark blue CDRs and the VL is coloured in magenta with orange CDRs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

system. After the primary ELISA screening, 199 specific hits were identified. Of these, 20 were sequenced that resulted in 15 unique Fab antibodies. All showed responses specific to the CCHFV antigen (Fig. 5). Three of these were taken forward for human IgG1 conversion: Ab38585ea-1, AbD38593ea-1 and AbD38595ea-1. Results from these showed specificity towards the antigenic target of two of the antibodies, with AbD38593ia-1 demonstrating the strongest responses whereas AbD38585ia-1 showed little sign of specific binding activity (Fig. 6).

3.5. Comparison of humanised antibodies from the two different approaches

Humanised antibodies towards recombinant CCHFV NP antigen had been generated through two independent routes: (i) via sequencing of a mouse hybridoma and (ii) using human combinatorial antibody libraries. A total of eight fully humanised antibodies were generated through these approaches and directly compared in an ELISA. Results demonstrated that those produced via the traditional route (cAb prefix), including the immunisation of mice with the antigen, outperformed those generated via an in vitro only approach (AbD prefix) (Fig. 7).

4. Discussion

A comparison of producing monoclonal humanised antibodies against CCHFV NP using two techniques has been undertaken. In this study we have shown that the use of mouse hybridomas gave the best performing antibodies, compared to the non-animal approach of HuCAL screening.

Our results are important in the context of a general movement away from the use of animals in research, where alternatives are being developed. The use of non-animal alternatives are a key element of the reduction theme of the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) (Burden et al., 2015). In addition, the UK Health Security Agency (UKHSA) is a signatory on the Concordat on Openness of Animal Research in the UK (Jarrett, 2016); championing the open reporting of animal studies. The finding that the non-animal alternative did not perform as effectively concurs with views from others, where a restriction on the immunisation of animals could significantly hamper research involving monoclonal antibodies at the current time (Bradbury et al., 2021). This conflicts with the recommendation of the European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) on non-animal -derived antibodies (Viegas Barroso et al., 2020) and the views of others who have argued that engineered antibodies are indistinguishable from those of animal-derived ones (Gray et al., 2020). Whilst we acknowledge that non-animal alternatives offer important replacement routes, for this application the HuCAL approach was suboptimal highlighting that further improvement is required. This supports the view that whilst display technologies have produced some beneficial drugs, their success is currently limited (Truppo et al., 2020) and in this case they are not at a level to substitute for established hybridoma technology (Gonzalez-Fernandez et al., 2020). Going forward, continued improvement of the HuCAL and recombinant technology continues apace. In addition, other non-animal approaches are also available. For example, mining the B cell repertoires from convalescent donors as has been used for identification of CCHFV glycoprotein-specific neutralising antibodies (Fels et al., 2021). Alternatively, transgenic mice that can express fully human

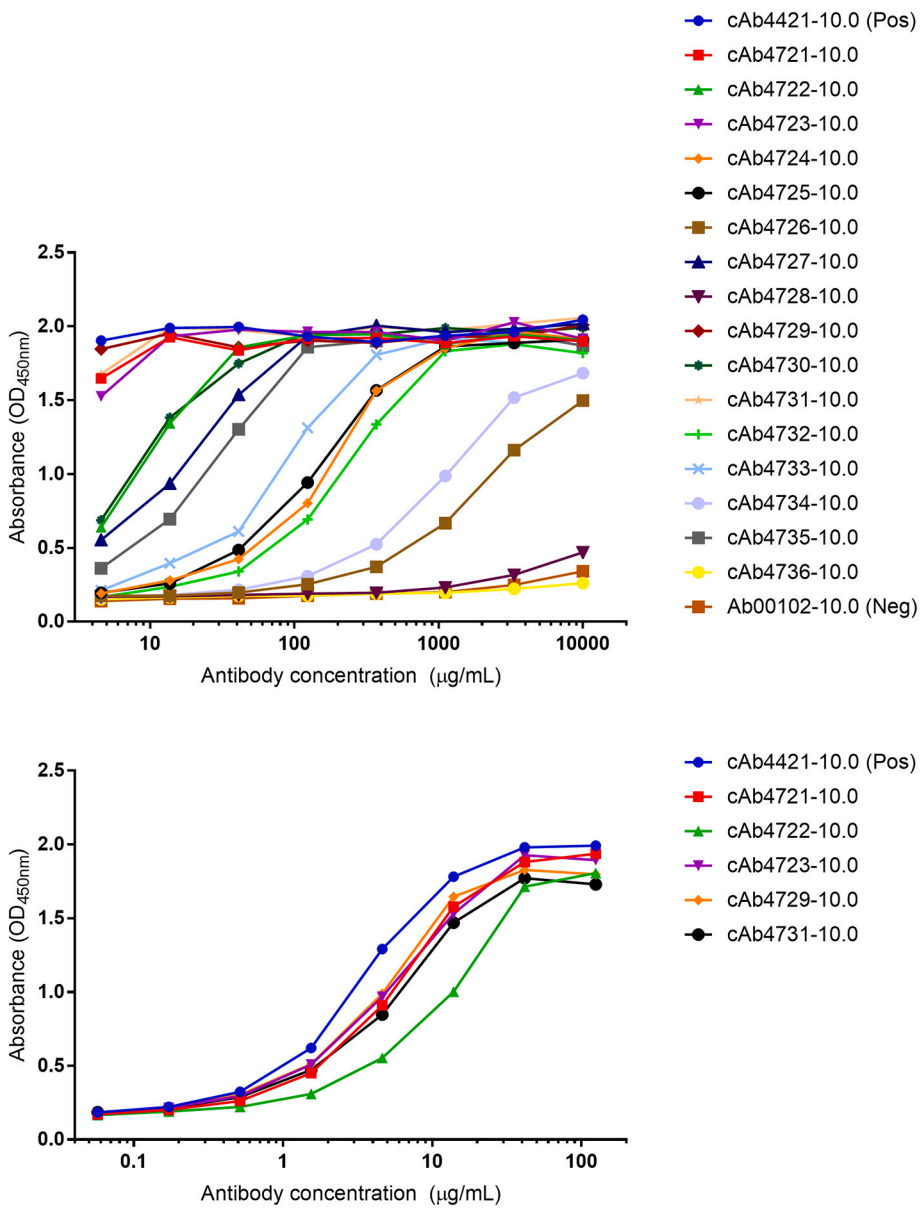

Fig. 4. Binding activities of humanised antibodies to recombinant CCHFV NP antigen. (a) Assessment of all 16 generated humanised antibodies. (b) Refined kinetics of the top five binding antibodies.

Table 3
Summary of the IC50 values for the chimeric antibody and top five humanised antibodies.

Antibody	IC50 (ng/ml)
Ab04421–10.0 (chimeric)	3.48
Ab04729–10.0	4.62
Ab04721–10.0	5.89
Ab04723–10.0	5.56
Ab04731–10.0	5.69
Ab04722–10.0	13.66

antibodies have also been developed and are increasing being used to develop therapeutic antibodies (Lu et al., 2020), although ultimately this latter approach does not reduce the reliance on the use of animals in the procedure.

The intended use of the humanised antibody specific to CCHFV NP is for development and standardisation of serological diagnostic assays. Although there are some commercially-available ELISA kits for the detection of CCHFV-specific antibodies, some are difficult to obtain or not offered to international customers (Mertens et al., 2013). The production and open availability of a humanised monoclonal antibody against CCHFV NP overcomes accessibility issues which has hampered development and standardisation of serology tests (Lombe et al., 2021). The majority of studies on CCHFV have been focused in specific endemic areas, especially Turkey but also Iran, Afghanistan, Pakistan and India (Belobo et al., 2021). Studies in other regions, particular in low-resource settings where the burden of CCHFV is likely higher, is therefore underrepresented; and so cost-effective, standardised assays may address this current shortfall.

The sequence definition contained herein allows synthesis ad infinitum (Bradbury et al., 2021). Whilst the humanisation was essential to ensure accurate use as a control and compatibility with human antibody-specific reagents, it might also have applicability for standardising kits for other species where Protein A/G can be substituted due to there being multiple IgG classes in most mammalian species (Patmawati et al., 2018). Whilst murine monoclonal antibodies against CCHFV NP have been reported previously, sequence information and

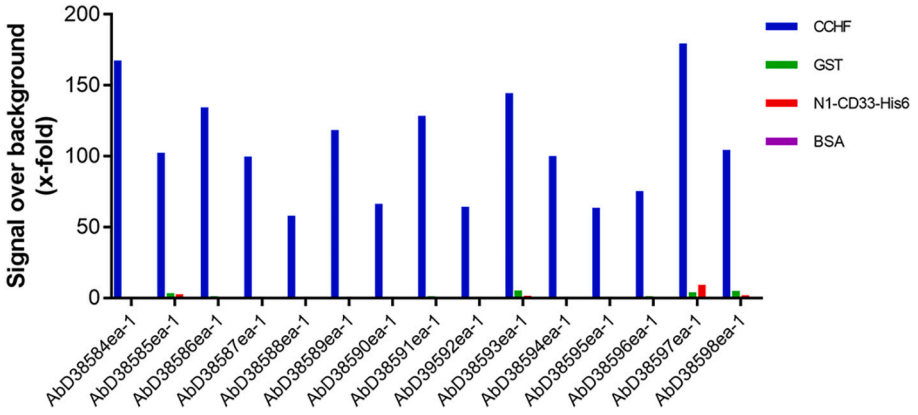

Fig. 5. Binding of unique Fab antibodies against recombinant CCHFV NP compared to three control antigens (glutathione S-transferase (GST), the ectodomain of human CD33 fused to the N1 domain of phase M13 (N1-CD33-His6) and bovine serum albumin (BSA)).

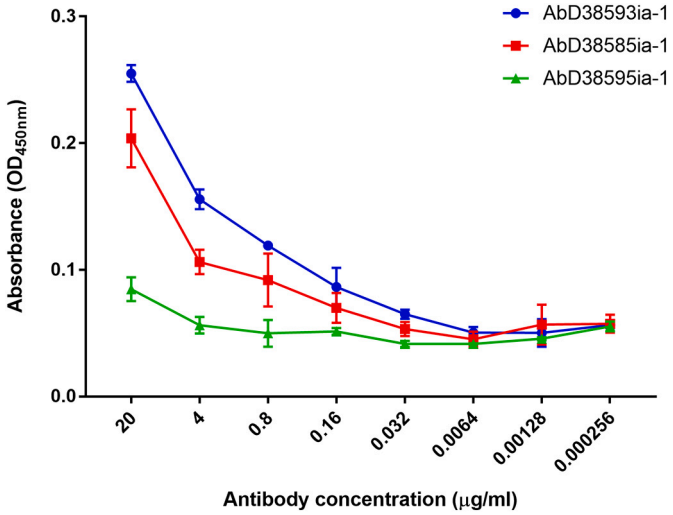

Fig. 6. Binding of three human IgG1 converted antibodies to recombinant CCHFV NP.

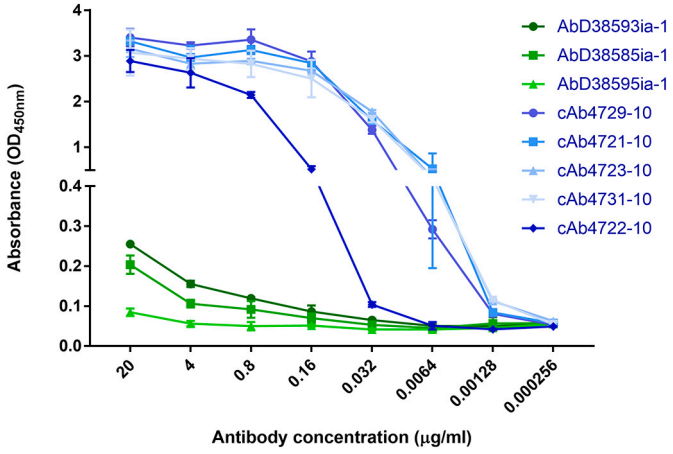

Fig. 7. Direct comparison of humanised antibody binding generated from two approaches: sequencing of a mouse hybridoma and use of a human antibody library.

humanisation was not performed (Schuster et al., 2016).

Whilst the primary purpose of our work was to establish a positive control for serology assays, the utility of the monoclonal antibodies produced may be expanded to other approaches including using antibody sandwich ELISA to detect the presence of CCHFV NP in samples as a diagnostic tool as used by others (Saijo et al., 2005; Shrivastava et al., 2021). In addition to recombinant NP as antigen for ELISA binding, a comparison of NP along with the mucin-like variable domain region (from GPC ORF) has been undertaken with inclusion of both proteins being able to differentiate acute and convalescence phases of CCHF disease (Gulce-Iz et al., 2021). Similar differences in performance of immunoassays have been reported in respect to the phase of infection samples were collected (Emmerich et al., 2021).

Due to the different - and often unpredictable – performance of the various platforms for monoclonal antibody production such as in vitro display systems, antibody libraries and individual animal variability, it is currently impossible to predict which technique will be the most successful (Bradbury et al., 2021). Whilst our experience is with one protein antigen (CCHFV NP), it is conceivable that other antigens may fare differently. With future investment and development of non-animal approaches, the antibody development landscape is changing rapidly and that in future a range of effective alternatives will exist.

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Declaration of Competing Interest

No potential conflict of interests are declared by any of the authors.

Data availability

Data will be made available on request.

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