Epidemiol. Infect. (2015), **143**, 1451–1456. © Cambridge University Press 2014
doi:10.1017/S0950268814002271

SHORT REPORT
Identification of human linear B-cell epitope sites on the envelope glycoproteins of Crimean-Congo haemorrhagic fever virus

D. GOEDHALS[1], J. T. PAWESKA[2,3] AND F. J. BURT[1]*

[1] *Department of Medical Microbiology and Virology, National Health Laboratory Service/University of the Free State, Bloemfontein, South Africa*
[2] *Center for Emerging and Zoonotic Pathogens, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, South Africa*
[3] *School of Pathology, Faculty of Health Sciences, University of the Witswatersrand, South Africa*

Received 24 April 2014; Final revision 14 July 2014; Accepted 9 August 2014; first published online 4 September 2014

SUMMARY

A peptide library was used to screen for regions containing potential linear B-cell epitope sites in the glycoproteins and nucleoprotein of Crimean-Congo haemorrhagic fever virus (CCHFV) in an enzyme-linked immunosorbent assay (ELISA). The library consisted of 156 peptides, spanning the nucleoprotein and mature G_N and G_C proteins in a 19-mer with 9-mer overlap format. Using pooled serum samples from convalescent patients to screen the library, six peptides were identified as potential epitope sites. Further testing of these six peptides with individual patient sera identified two of these peptides as probable epitope sites, with peptide $G_{1451–1469}$ reacting to 13/15 and peptide $G_{1613–1631}$ to 14/15 human sera. These peptides are situated on the G_C protein at amino acid positions 1451–1469 (relative to CCHFV isolate SPU103/97) (TCTGCYACSSGISCKVRIH) and 1613–1631 (FMFGWRILFCFKCCRRTRG). Identified peptides may have application in ELISA for diagnostic or serosurveillance purposes.

Key words: Arboviruses, bunyaviruses, emerging infections, viral haemorrhagic fever.

Crimean-Congo haemorrhagic fever virus (CCHFV) is a tick-borne virus belonging to the family Bunyaviridae [1]. The negative-stranded RNA genome consists of three segments (S, M, L) coding for the nucleoprotein, the envelope glycoproteins (G_N and G_C) and the viral polymerase, respectively [1]. The emergence of CCHFV in recent years in Southeast Europe and Southwest Asia highlighted the importance of developing serological assays for both diagnostic and serosurveillance purposes [2]. Although the preparation of CCHFV antigens from cell cultures or inoculation of suckling mice have been used, these techniques require the use of a biosafety level 4 laboratory which is not widely available and therefore limits the diagnostic and surveillance capabilities of many laboratories in endemic areas. Expression of recombinant CCHFV nucleoprotein antigens in baculovirus, mammalian and bacterial systems have also been utilized and have potential for application in detection assays [3–5]. Glycoproteins are more difficult to express, although by analogy with other bunyaviruses the glycoproteins should have application in detection assays.

Peptide libraries have been used to identify antigenic regions of CCHFV nucleoprotein, but epitopic regions of the glycoproteins have not previously been defined [6]. B-cell epitopes are classified as either conformational

* Author for correspondence: Professor F. J. Burt, Department of Medical Microbiology and Virology, Faculty of Health Sciences, National Health Laboratory Service/University of the Free State, Bloemfontein, 9301, South Africa.
(Email: burtfj@ufs.ac.za)

(discontinuous) or non-conformational (linear/continuous). Linear epitopes consist of short peptide fragments which are contiguous in the primary amino acid sequence, while conformational epitopes are a set of amino acids that are brought into proximity by protein folding but are not contiguous in the primary sequence [7, 8]. As antigen processing is not involved in immune recognition by B cells, these epitopes are often located on surface structures, are generally hydrophilic and are usually 4–8 amino acids in length [8]. In the present study, we screened for linear B-cell epitope regions in the nucleoprotein and envelope glycoproteins of CCHFV using a synthetic peptide library and sera from CCHF survivors in an enzyme-linked immunosorbent assay (ELISA) format.

An overlapping peptide library consisting of 156 peptides (19-mers with a 9-mer overlap) spanning the 482 amino acids of the nucleoprotein, the 292 amino acids of the mature G_N and the 648 amino acids of the mature G_C were synthesized (Mimotopes, Australia) based on the deduced amino acid sequences of CCHFV isolate SPU103/87 (GenBank accession numbers DQ211647 and DQ211634). The peptide length was selected to reduce the cost of the library production while allowing coverage of the complete genes for the major structural proteins of CCHFV. A peptide ELISA was developed using the synthetic peptides to screen for linear B-cell epitope regions using the serum of survivors of CCHF infection. Polysorb plates (Nunc, Denmark) were coated with 100 µl/well of 20 µg/ml peptide in 0·2 M carbonate buffer (pH 9·6) overnight at 4 °C. The following day, plates were blocked with 10% skimmed milk in carbonate buffer for 2 h at room temperature (22–24 °C). The plates were then washed using 0·05% Tween-20 in phosphate buffered saline (PBS, pH 7·2). Human sera diluted 1:100 in 10% skimmed milk in PBS were then added and incubated for 1 h at 37 °C. After washing, 100 µl anti-human IgG horseradish peroxidase (HRP) conjugate (Zymed, USA) at a dilution of 1:1000 in 10% skimmed milk in PBS was added to each well and incubated for 1 h at 37 °C. After further washing, plates were incubated with azino di-ethyl-benzothiazoline-sulfonic acid (ABTS®) peroxidase substrate (Kirkegaard and Perry Laboratories, KPL, USA) for 40 min at room temperature in the dark and optical density (OD) values were read at 405 nm/620 nm. All assays were performed in duplicate.

For screening the full peptide library of 156 peptides, three pools of sera were generated to yield two positive and one negative pool each comprised of nine sera. The positive sera derived from survivors of previous infection with CCHFV as confirmed by ELISA or reverse transcription–polymerase chain reaction testing performed at the National Institute for Communicable Diseases, Johannesburg, while the negative sera derived from volunteers with no history of CCHFV infection, exposure to CCHFV or risk factors for such exposure. Six peptides derived from the envelope glycoproteins reacted to both positive pools but not the negative pool; however, no peptides in the nucleoprotein were found to be reactive to the pooled sera (data not shown). The details of the reactive peptides, as well as specific OD values obtained with each pool are shown in Table 1. Five peptides were located on the G_C protein, with one reactive peptide on the G_N protein.

The six reactive peptides were then screened using individual sera from 15 survivors of previous CCHFV infection and three negative volunteers. The cut-off for each peptide was calculated using the mean of the negative sera plus 2 standard deviations. Peptides $G_{1451–1469}$ and $G_{1613–1631}$ were identified as including a probable linear B-cell epitope site with 13/15 and 14/15 sera showing positive OD results, respectively (Table 2). Both of these peptides are located on the G_C envelope glycoprotein. The OD values of the positive sera obtained with peptide $G_{1451–1469}$ ranged from 0·385 to 0·931 (cut-off 0·352), while the OD values for peptide $G_{1613–1631}$ ranged from 0·408 to 1·257 (cut-off 0·317). Peptides $G_{1181–1199}$ and $G_{1622–1640}$ each reacted with 7/15 sera. These peptides showed lower reactivity compared to peptides $G_{1451–1469}$ and $G_{1613–1631}$, with OD values for positive sera obtained with peptide $G_{1181–1199}$ ranging from 0·318 to 0·576 (cut-off 0·317) and with peptide $G_{1622–1640}$ from 0·474 to 0·720 (cut-off 0·381). None of the sera reacted to peptide $G_{1172–1190}$ and only two sera had OD values above the cut-off determined for peptide $G_{669–687}$.

The two sera (VBD49/10 and VBD10/09) which did not react to peptides $G_{1451–1469}$ and $G_{1613–1631}$ also failed to react to any of the other four peptides tested against the individual sera. As no further samples were available from these two patients at other time points, it could not be confirmed whether this reflects true lack of reactivity to all six peptides or whether sample integrity was compromised. However, sample VBD42/10 showed a low OD value on antibody ELISA testing in the laboratory, with an antibody titre of 1:100. The lack of reactivity to the peptides tested could possibly be based on a low-titre antibody

https://doi.org/10.1017/S0950268814002271 Published online by Cambridge University Press

Table 1. *Details of the six reactive peptides selected for further testing based on OD values obtained in the peptide ELISA using one negative and two positive serum pools. Two overlapping peptide pairs are noted with each pair probably representing a single epitopic region as indicated with a superscript numeral. Amino acid residues in the overlapping region are underlined*

Peptide number	Peptide	Amino acid position relative to strain DQ211634		Protein	Positive pool 1 OD values	Positive pool 2 OD values	Negative pool OD values
		Start	End				
$G_{669–687}$	KIPLLGKMAIYICRMSNHP	669	687	G_N	0·845	0·407	0·152
$G_{1172–1190}$[1]	RCGCTSSTCLHKEWPHSRN	1172	1190	G_C	1·038	0·583	0·241
$G_{1181–1199}$[1]	LHKEWPHSRNWRCNPTWCW	1181	1199	G_C	0·803	1·073	0·170
$G_{1451–1469}$	TCTGCYACSSGISCKVRIH	1451	1469	G_C	1·434	0·811	0·305
$G_{1613–1631}$[2]	FMFGWRILFCFKCCRRTRG	1613	1631	G_C	0·871	0·574	0·174
$G_{1622–1640}$[2]	CFKCCRRTRGLFKYRHLKD	1622	1640	G_C	0·874	0·544	0·189

rather than due to compromised sample integrity. Sample VBD10/09 was collected from a patient about 3 months after the onset of illness. The only other patient sampled at a similar time point showed relatively weak reactivity for peptides $G_{1451–1469}$ and $G_{1613–1631}$ and negative results for the remaining peptides. The remainder of the samples in the panel were obtained between 11 months and 11 years post-infection. It is known that epitope recognition varies with IgM and IgG class switching and with affinity maturation of the IgG response to viral infections [9, 10]. As the majority of the serum samples included in the pools used to screen the peptide library were obtained at least 1 year post-infection, it is possible that the six peptides identified in the initial ELISA screen are better recognized by antibodies in the later convalescent phase when extensive affinity maturation of IgG responses has occurred. Although acute phase and early convalescent phase serum panels were not available in the current study, it would be of interest to evaluate differences in peptide recognition at these time-points after infection with CCHFV, particularly if the peptide ELISA were to be developed as a diagnostic assay for use in the acute phase of infection.

Sequence data for CCHFV isolates from patients sampled in this study were unavailable. Conservation of predicted epitopic regions was determined using complete genome sequence data for southern African isolates. Complete genome sequence data determined in our laboratory for 10 southern African isolates as well as four complete genomes from South African CCHFV isolates available in GenBank were used to determine the conservation of the epitopic regions identified (Fig. 1). The deduced amino acid sequences for peptide $G_{1613–1631}$ show that this region is highly conserved with one isolate showing a single amino acid difference. Similarly, four isolates showed a single amino acid difference for peptide $G_{1451–1469}$ compared to isolate SPU103/87 which was used as the reference sequence for the construction of the peptide library. Peptides $G_{1172–1190}$ and $G_{1181–1199}$ showed 100% conservation in amino acid sequence, while peptides $G_{669–687}$ and $G_{1622–1640}$ showed a maximum of two amino acid differences between isolates. All amino acid substitutions were conserved in polarity. The lack of reactivity to the individual patient sera by these peptides cannot therefore be explained by amino acid diversity, as all of the peptides found to be reactive in the screening ELISA were well conserved. In order to predict potential cross-reactivity of peptides $G_{1451–1469}$ and $G_{1613–1631}$ against other viruses, the peptide sequences were subjected to Basic Local Alignment Search Tool (BLAST) analysis using the protein–protein BLAST search (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST search against peptide $G_{1451–1469}$ recognized only CCHFV glycoprotein with query coverage and identity >80%, implying that the specificity of this peptide is likely to be high. The search against peptide $G_{1613–1631}$ returned hits for Hazara virus, Dugbe virus and Nairobi sheep disease virus glycoproteins

https://doi.org/10.1017/S0950268814002271 Published online by Cambridge University Press

Table 2. *Results of two reactive peptides tested with sera from 15 patients with previous CCHFV infection indicating optical density (OD) values obtained, as well as the approximate duration from time of illness to sample collection. All OD values for CCHF survivors represent the mean of values obtained in duplicate testing. OD values above the relevant cut-off are underlined*

Laboratory number	Approximate duration since illness	Peptide $G_{1451–1469}$ OD value	Peptide $G_{1613–1631}$ OD value
10/09	3 months	0·092	0·115
20/13	3 months	0·385	0·408
38/11	8 months	0·499	0·733
40/11	8 months	0·478	0·720
06/11	11 months	0·632	0·970
51/10	2 years	0·714	1·013
54/11	2 years	0·457	0·671
42/10	2·5 years	0·200	0·324
49/11	3 years	0·428	0·812
51/11	5·5 years	0·411	0·591
39/11	7 years	0·636	1·173
41/10	8·5 years	0·717	0·868
30/10	10 years	0·931	1·210
52/10	10 years	0·846	1·257
43/11	11 years	0·525	1·100
Negative	n.a.	0·184	0·189
Cut-off	n.a.	0·352	0·317

n.a., Not applicable.

although the coverage and identity were all <80%. Further laboratory testing is required to determine whether this would lead to significant cross-reactivity and therefore lower the sensitivity of an assay based on this peptide.

The predicted amino acid sequences used to generate the peptide library were analysed using the Immune Epitope Database (IEDB) Analysis Resource B Cell Epitope Prediction Tools and compared to the epitopes identified by the screening ELISA. Although peptide $G_{669–687}$ located on the G_N protein, was identified by Kolaskar and Tongaonkar Antigenicity software as a predicted protein antigen (threshold setting = 1·000) [11], only four amino acids in the peptide were identified as a possible epitope by Bepipred Linear Epitope Prediction software (threshold setting = 0·350) [12]. Regions of peptides $G_{1172–1190}$, $G_{1181–1199}$, $G_{1451–1469}$, $G_{1613–1631}$ and $G_{1622–1640}$ on the G_C protein were all identified as potential antigens by Kolaskar and Tongaonkar Antigenicity software, while Bepipred Linear Epitope Prediction software identified only peptides $G_{1172–1190}$ and $G_{1181–1199}$. Parker Hydrophilicity Prediction was also performed and identified peptides $G_{1172–1190}$ and $G_{1451–1469}$ as hydrophilic peptides and therefore potentially epitopic with scores of 2·937 and 2·074, respectively (threshold setting = 1·290) [13]. Peptides $G_{669–687}$, $G_{1181–1199}$, $G_{1613–1631}$ and $G_{1622–1640}$ were identified as hydrophobic, with scores of −0·426, 0·505, −1·311 and 0·179 respectively. As linear B-cell epitopes are mostly hydrophilic, the strongly hydrophobic score of peptide $G_{1613–1631}$ emphasizes the necessity of functional laboratory testing to support predictive software. As the precise epitopic region of peptide $G_{1613–1631}$ was not determined within the 19-mer, the true hydrophilicity score of this epitope could not be determined and may differ from the overall score for the peptide.

As the aim of this study was to identify widely recognized epitopes for possible use in serological assays rather than the complete elucidation of linear B-cell epitope sites of CCHFV, the use of serum pools to screen the complete peptide library represented a practical approach given the size of the peptide library. Epitopes recognized at a low frequency in survivors of CCHFV infection may, however, not have been detected. The efficacy of synthetic peptides in immunoassays is influenced by their ability to bind to solid surfaces, the fact that antigenicity may be lost when binding to solid surfaces due to antigenic side-chains not being accessible, and differing solubility [14]. In a previous study, an epitopic site in the CCHFV nucleoprotein was identified using 8-mer overlapping peptide libraries [6]. The peptide used in the previous study was of higher purity (95%) and was biotinylated and therefore bound to the surface via neutravidin. This technique significantly enhances binding properties and site accessibility which can be hidden when peptides are bound directly to a solid phase. As these characteristics cannot be reliably predicted, laboratory testing of peptide libraries or peptides based on prediction software is required to identify peptides with good binding ability which retain antigenicity in an immunoassay, such as the two peptides identified in this study. Unbiotinylated 19-mer peptides were used in the current study as they were primarily selected for ELISPOT assays for the study of T-cell responses in CCHFV survivors, for which biotinylated peptides are not used. The ease with which peptides are manufactured and their stability are advantageous in a diagnostic setting. A combination of peptides may be required to ensure adequate sensitivity for the detection of IgM and IgG.

https://doi.org/10.1017/S0950268814002271 Published online by Cambridge University Press

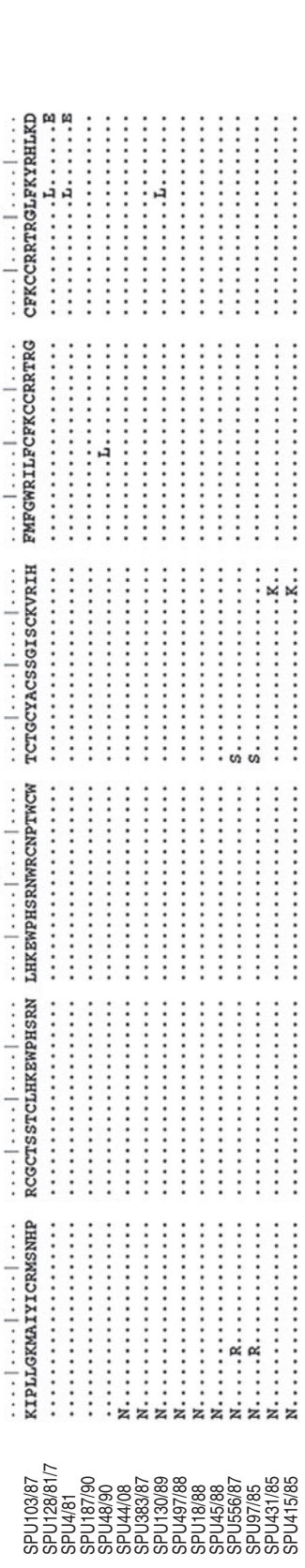

Fig. 1. Alignment of amino acid sequences of reference SPU103/87 with 14 southern African CCHFV isolates illustrating conservation of peptides $G_{669–687}$, $G_{1172–1190}$, $G_{1181–1199}$, $G_{1451–1469}$, $G_{1613–1631}$ and $G_{1622–1640}$.

Humoral antibody responses to the nucleoprotein and glycoproteins can be detected following infection with CCHFV from about day 7 onwards after disease onset. While IgM responses generally decline to undetectable levels by 4–6 months post-infection, IgG responses are detectable for at least 10 years [6]. In the current study, IgG responses to epitopes on the G_C glycoprotein were confirmed at least 11 years after infection. The envelope glycoproteins are responsible for binding of CCHFV to cell receptors and are targets for neutralizing antibodies which have been shown to protect mice in passive-immunization experiments [15]. Recombinant nucleoproteins are usually targeted for development of detection assays because they are more conserved and recombinant glycoproteins are difficult to express. However peptides mimicking epitopic or immunodominant regions from conserved regions of the glycoprotein may be useful for serological assay development particularly for serosurveillance which is important for monitoring disease endemicity. Peptide-based ELISA assays have previously been used for the diagnosis and serosurveillance of diverse organisms including hepatitis A virus, monkeypox virus and *Trypanosoma cruzi* [16–18]. We identified two potential linear B-cell epitope sites on the G_C protein. Further investigation using shorter peptides would enable identification of specific epitopic sites within the peptides. Validation of the two peptides using larger numbers of serum samples would determine the usefulness of these peptides in serological assays.

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. Informed consent was obtained from each study participant (ECUFS NR 152/06).

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of South Africa and the Polio Research Foundation, South Africa.

DECLARATION OF INTEREST

None.

REFERENCES

1. **Clerx JP, Casals J, Bishop DH.** Structural characteristics of nairoviruses (genus *Nairovirus*, Bunyaviridae). *Journal of General Virology* 1981; **55**: 165–178.

2. **Maltezou HC, Andonova L, Andraghetti R.** Crimean-Congo hemorrhagic fever in Europe: current situation calls for preparedness. *Eurosurveillance* 2010; **15**: 19504–19508.
3. **Dowall SD, *et al.*** Development of an indirect ELISA method for the paralled measurement of IgG and IgM antibodies against Crimean-Congo haemorrhagic fever (CCHF) virus using recombinant nucleoprotein as antigen. *Journal of Virological Methods* 2012; **179**: 335–341.
4. **Saijo M, *et al.*** Immunofluorescence technique using HeLa cells expressing recombinant nucleoprotein for detection of immunoglobulin G antibodies to Crimean-Congo hermorrhagic fever virus. *Journal of Clinical Microbiology* 2002; **40**: 372–375.
5. **Samudzi RR, *et al.*** Bacterial expression of Crimean-Congo hemorrhagic fever virus nucleoprotein and its evaluation as a diagnostic reagent in an indirect ELISA. *Journal of Virological Methods* 2012; **179**: 70–76.
6. **Burt FJ, *et al.*** Human defined antigenic region on the nucleoprotein of Crimean-Congo hemorrhagic fever virus identified using truncated proteins and a bioinformatics approach. *Journal of Virological Methods* 2013; **193**: 706–712.
7. **Peters B, *et al.*** The design and implementation of the immune epitope database and analysis resource. *Immunogenetics* 2005; **57**: 326–336.
8. **Bottino CC, *et al.*** Chagas disease-specific antigens: characterization of epitopes in CRA/FRA by synthetic peptide mapping and evaluation by ELISA-peptide assay. *BMC Infectious Diseases* 2013; **13**: 568.
9. **Gao F, *et al.*** Enterovirus 71 viral capsid protein linear epitopes: identification and characterization. *Virology Journal* 2012; **9**: 26.
10. **Leonova IV, *et al.*** Development of human antibodies against linear antigenic and immunogenic regions of respiratory syncytial virus (RSV) nucleocapsid and phosphor-proteins shows the site-directed characteristics. *Journal of Clinical Virology* 1998; **11**: 137–147.
11. **Kolaskar AS, Tongaonkar PC.** A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Letters* 1990; **276**: 172–174.
12. **Larsen JEP, Lund O, Nielsen M.** Improved methods for predicting linear B-cell epitopes. *Immunome Research* 2006; **2**: 2.
13. **Parker JM, Guo D, Hodges RS.** New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and X-ray-derived accessible sites. *Biochemistry* 1986; **25**: 5425–5432.
14. **Tam JP.** Recent advances in multiple antigen peptides. *Journal of Immunological Methods* 1996; **196**: 17–32.
15. **Bertolotti-Ciarlet A, *et al.*** Cellular localization and antigenic characterization of Crimean-Congo hemorrhagic fever virus glycoproteins. *Journal of Virology* 2005; **79**: 6152–6161.
16. **Dubois ME, Hammarlund E, Slifka MK.** Optimization of peptide-based ELISA for serological diagnotics: a retrospective study of human monkeypox infection. *Vector Borne and Zoonotic Diseases* 2012; **12**: 400–409.
17. **Gómara MJ, *et al.*** Use of linear and multiple antigenic peptides in the immunodiagnosis of acute hepatitis A virus infection. *Journal of Immunological Methods* 2000; **234**: 23–34.
18. **Saba ES, *et al.*** Anti-Trypanosoma cruzi cross-reactive antibodies detected at high rate in non-exposed individuals living in non-endemic regions : seroprevalence and association to other viral serologies. *PLoS ONE* 2013; **8**: e74493.