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Human defined antigenic region on the nucleoprotein of Crimean-Congo hemorrhagic fever virus identified using truncated proteins and a bioinformatics approach

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Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne viral zoonosis widely distributed in Africa, Asia and eastern Europe. In this study, amino acid sequence data for the CCHFV nucleoprotein (NP) was used to identify potential linear epitopic regions which were subsequently included in the design of large and small truncated recombinant NP antigens and peptide libraries. Two truncated recombinant CCHFV NP antigens were prepared based on results of prediction studies to include epitopic regions and exclude hydrophobic regions that could influence protein expression and solubility. Serum samples were collected from acute and convalescent patients. An IgG antibody response was detected in 16/16 samples tested using the large recombinant NP-based ELISA and in 2/16 using the small recombinant NP-based ELISA. A total of 60 peptides covering predicted epitopic regions of the NP were synthesized and peptide NRGGDENPRGPVSR at amino acid position 182–195, reacted with 13/16 human serum samples. In summary, functional assays are required to determine the biological activity of predicted epitopes for development of peptide based assays for antibody detection. Bacterially expressed complete NP antigens have previously been shown to be useful tools for antibody detection. Truncation of the antigen to remove the hydrophobic C terminus had no impact on the ability of the antigen to detect IgG antibody in human sera. The results indicate that the region from amino acids 123 to 396 includes a highly antigenic region of the NP with application in development of antibody detection assays.

1. Introduction

Crimean-Congo hemorrhagic fever virus (CCHFV) is a tick-borne viral zoonotic agent widely present in Africa, Asia and eastern Europe within the distribution range of ticks belonging to the genus *Hyalomma* (Hoogstraal, 1979). CCHFV belongs to the genus *Nairovirus* within the family *Bunyaviridae* and is a negative-stranded RNA virus with a tripartite genome (Clerx et al., 1981). The three genome segments S (small), M (medium) and L (large) encode the virus nucleocapsid protein (NP), two envelope proteins (G_N and G_C) and L viral transcriptase proteins, respectively (Clerx et al., 1981; Sanchez et al., 2002).

CCHFV is transmitted to humans by tick-bite or by contact with blood or tissues from infected patients or livestock. The fatality rate in southern Africa is approximately 30% (Burt et al., 2009). The emergence and re-emergence of CCHFV emphasizes the importance of human and veterinary surveillance and developing diagnostic capacity which requires the development of standardized, rapid, sensitive and specific assays (Maltezou et al., 2010). Antibody detection is usually performed by ELISA or immunofluorescent assays which currently depend on the preparation of antigen from infected cell cultures or from inoculation of suckling mice and the subsequent preparation of antigen from mouse brain tissue. Both techniques require biosafety level (BSL) 4 facilities for culturing CCHFV. Identification of immunodominant regions and epitopic regions on viral proteins could play a role in developing novel serological assays for detecting immune responses. The complete open reading frame from the S gene of CCHFV was previously codon optimized and the NP expressed in *Escherichia coli* from the synthetic gene (Samudzi et al., 2012). The recombinant antigen was able to detect IgG antibody in acute and convalescent sera.

Identification of specific epitopic sites will contribute to development of diagnostic assays. There are various methods available for mapping epitopes. Antibody defined epitopes are classified

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as linear (non-conformational) or discontinuous (conformational) and are primarily composed of a single stretch of the polypeptide chain. Discontinuous epitopes are composed of different parts of the polypeptide chain that are brought into close proximity by the folding of the protein. Linear epitopes comprise of approximately 10% of all epitopes, where the linear peptide fragment of the epitope cross-reacts with the corresponding antibodies (Pellequer et al., 1991). Peptide libraries can be used to identify linear epitopes however they are costly. Databases are available for predicting linear epitopic regions based on characteristics of individual amino acids. Parameters such as hydrophilicity, polarity and antigenic propensity of polypeptide chains have been correlated with the location of epitopes. All prediction calculations are based on propensity scales. Prediction of potentially immunogenic epitopes in a given protein may reduce experimental efforts in determining epitopes needed for vaccine development and immunodiagnostics. However, the correlation between prediction and biological activity requires investigation.

The NP of CCHFV is known to be highly immunogenic and an abundant viral protein hence we selected to perform an analysis of the NP and determine the role of prediction software in identification of epitopic sites. Truncated recombinant CCHFV NP antigens were prepared based on results of prediction studies to include epitopic regions and exclude hydrophobic regions that could influence protein solubility. The truncated proteins were used to identify regions of the protein important for inducing detectable antibody responses. This study aimed at using truncated recombinant NP and epitope predication software to identify regions of the NP that induce antibody responses in humans with potential application in detection assays.

2. Material and methods

2.1. Epitope prediction and hydrophilicity

Sequence data for 37 CCHFV isolates, representing geographically distinct regions, were retrieved from GenBank. For each isolate the predicted amino acid sequence for the CCHFV NP was analyzed using Bepipred Epitope Prediction Software and Parker Hydrophilicity Prediction (accessible at: Immune Epitope Database and analysis Resource www.immunoepitope.com). A recombinant NP has previously been shown in our laboratory to react with human serum samples from South African patients (Samudzi et al., 2012). To determine the significance of predicted sites, recombinant truncated NP antigens were prepared which included predicted antigenic and epitopic regions. To identify epitopes, peptide libraries covering predicted epitopic regions were synthesized.

2.2. Truncated recombinant NP antigens

Optimization of the gene from isolate SPU 415/85 encoding the CCHFV NP was performed using OptimumGene algorithm software from GenScript (New Jersey, USA) as described previously and the optimized gene was synthesized and cloned into pUC57 with *Bam*H1 and *Pst*1 restriction site modifications at the 5′ end and 3′ end, respectively (Samudzi et al., 2012). The complete NP includes three significant hydrophilic peaks. The ORF was truncated to include regions encoding for hydrophilic and predicted epitopic sites and the recombinant proteins were expressed in a bacterial system. Briefly, truncated regions of the synthesized gene were amplified using the following primer pairs: primers F1 and R1 amplified a 369 base pair region and primers F1 and R3 amplified an 1188 base pair region (as illustrated in Fig. 1).

F1 5′ **GCC GGA TCC** GAA AAC AAA ATC GAA GTG AAC AAC AAA G 3′

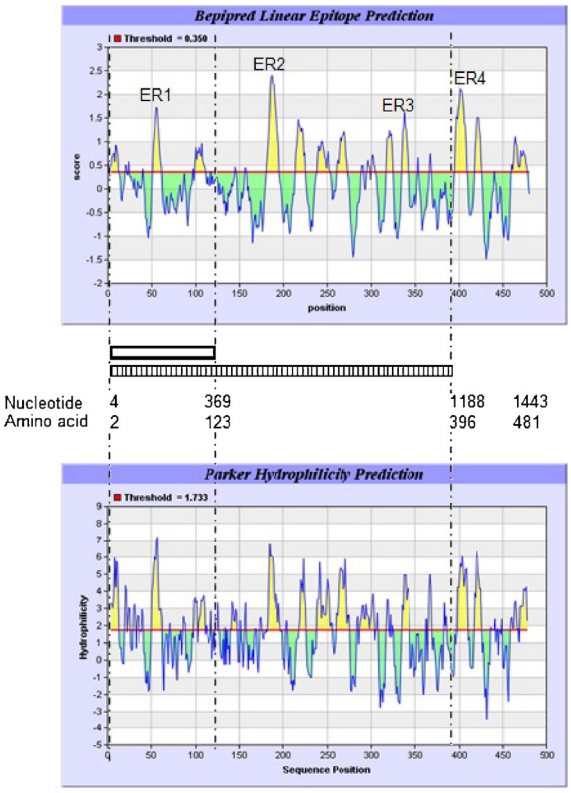

Fig. 1. Analysis of representative CCHFV strain. Prediction software was used to predict linear epitopic regions and hydrophilic regions which were aligned with truncated recombinant proteins. ER1-4 represent potential epitopic regions based on highest score.

R1 5′ **GCC CTG CAG** TCA CGC CAG CTG TTC AAT TTT CG 3′
R3 5′ **GCC CTG CAG** TCA CAC CGG GAT GGT ACC AAA G 3′

The 5′ ends of each primer (indicated in bold) were modified to include suitable restriction sites to facilitate cloning into the expression vector. Each amplicon was cloned into pCOLD-TF (Takara Bio, Paris, France) generating constructs designated pCOLD-TF-CCHFVNP369 and pCOLD-TF-CCHFVNP1188. The pCOLD-TF-CCHFVNP1188 and pCOLD-TF-CCHFVNP369 constructs were used to transform *E. coli* OverExpress C43 (DE3) (Overexpress) competent cells (Lucigen, Wisconsin, USA) and the truncated recombinant proteins were designated NP396 and NP123 respectively. A mock antigen was prepared using cells transformed with pCold-TF plasmid lacking the CCHFV NP gene. The *E. coli* transformants were propagated in 5 ml Luria Bertani (LB) broth containing 100 μg/ml ampicillin and incubated overnight at 37 °C with shaking at 200 rpm. A 2 ml aliquot of the overnight culture was inoculated into 40 ml of LB medium containing ampicillin and incubated at 25 °C with shaking until an optical density (OD) reading at 600 nm between 0.4 and 0.5 was reached. The bacterial culture was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown for 24 h with shaking at 16 °C. The bacterial culture was harvested and the cells clarified. The cells were lysed by resuspending the pellet in Bugbuster Protein Extraction Reagent (Novagen, Darmstadt, Germany) at a final concentration of 200 mg/ml. An aliquot of r-Lysozyme (Novagen, San Diego, USA) was added to give a final concentration of 1 mg/ml and 50 units/ml of benzonase (Novagen, San Diego, USA) were added to the cell suspension. The cell suspension was sonicated and after clarification, the recombinant CCHFV NP fusion proteins containing a 6x His tag were purified from the soluble fraction using Protino Ni-TED resin according to the manufacturer's instructions for purification under native

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