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Fine mapping epitope on glycoprotein Gc from Crimean-Congo hemorrhagic fever virus

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ABSTRACT

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne zoonosis, caused by CCHF virus (CCHFV) and which there are no diagnostic or therapeutic strategies. The C-terminus of glycoprotein (Gc) encoded by the CCHFV M gene is responsible for CCHFV binding to cellular receptors and acts as a neutralizing-antibody target. In this study, a modified biosynthetic peptide technique (BSP) was used to identify fine epitopes of Gc from the CCHFV YL04057 strain using rabbit antiserum against CCHFV-Gc. Six B cell epitopes (BCEs) and one antigenic peptide (AP) were identified: E1 (^{88}VEDASES^{94}), E2 (^{117}GDRQVEE^{123}), E3 (^{241}EIVTLH^{246}), AP-4 (^{281}DFQVYHVGNLLRGDKV^{296}), E5a (^{370}GDTP QLDL^{377}), E5b (^{373}PQLDLKAR^{380}), and E6 (^{443}HVRSSD^{448}). Western blotting analysis showed that each epitope interacted with the positive serum of sheep that had been naturally infected with CCHFV, and the results were consistent with that of Dot-ELISA. The multiple sequence alignment (MSA) revealed high conservation of the identified epitopes among ten CCHFV strains from different areas, except for epitopes AP-4 and E6. Furthermore, three-dimensional structural modeling showed that all identified epitopes were located on the surface of the Gc "head" domain. These mapped epitopes of the CCHFV Gc would provide a basis for further increase our understanding CCHFV glycoprotein function and the development of a CCHFV epitope-based diagnostics vaccine and detection antigen.

1. Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne zoonosis that caused by Crimean-Congo hemorrhagic fever virus (CCHFV), which is widely distributed in more than 30 countries and distinct regions poses a serious threat to human health, with fatality rate of up to 30–50% [1,2]. Handling of the infectious CCHF virus requires biosafety level 4 (BSL4) containment [3]. The first case of CCHF in China was reported in Bachu county of Xinjiang in 1965, and since then, there have been several outbreaks in southern Xinjiang causing serious infections. Several regions in the Tarim Basin and the Junggar Basin were identified as natural epidemic foci of CCHF [4]. Currently, there is no applicable diagnostic or therapeutic approach for this disease.

CCHFV belongs to the genus *Nairovirus*, in the family of *Bunyaviridae*, and the CCHFV M gene encodes the viral envelope glycoprotein (GP) (including mature N- terminal glycoprotein Gn and C-terminal glycoprotein Gc) [5]. Bertolotti et al. [6] reported that envelope glycoprotein Gc is responsible for CCHFV binding to cellular receptors and acts as a neutralizing-antibody target capable of neutralization in mammalian cells, resulting in protection of mice in passive-immunization experiments. Additionally, Xiao et al. [7] found that Gc interacts with the surface nucleolin of the host cell. Therefore, it is important to study the epitope or immune-dominant region of the conserved region of glycoprotein Gc for the development of CCHFV detection and vaccine [8].

Several studies have demonstrated multiple methods and techniques, including the use of recombinant DNA, peptide synthesis, and protein-display libraries for epitope mapping [9–12]. The method based on modification of biosynthetic peptides involves expression of a 15- to 25-amino acid (aa) peptide fragment to allow determination of whether

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the peptide fragment contains a binding region using a monoclonal or polyclonal antibodies (pAbs) via western blot. For fine epitope motif mapping the positive dominant regions were identified by using biosynthetic peptides method (BSP), eight overlapping peptides were further designed and expressed, then followed by immunoblotting to allow epitope mapping. This method is simple, cost efficient, reliable, and adaptable, as well as particularly well-suited for identifying fine epitopes [13]. A series of studies used this method to successfully identify the minimally sized epitope motifs capable of recognition by human zonapellucida protein [14], pristine ruminant epidemic virus N protein [15], and human papillomavirus (HPV) E6 and E7 proteins [16], thereby providing a foundation for research into HPV-preventive vaccines. Additionally, Yu et al. [17] used this method to finely map the B cell epitopes (BCEs) on Peste des petits ruminants virus nucleocapsid protein (NP), subsequently identifying 19 linear BCEs. Luan et al. [18] used this method to perform linear epitope scanning of the foot-and-mouth disease virus VP4 protein, ultimately identifying the conserved epitope motif INNYYM. Liu et al. [19] identified the five BCEs recognized by multiple pAbs on the middle segment of CCHFV nucleoprotein $NP^{237-305}$ using this method. These findings suggest the feasibility of this method for epitope identification.

In our previous study, eight epitopes on the Gn of CCHFV were identified using pAbs against recombinant (r)-Gn combined with the BSP method [20]. However, there have only been a few reports on epitope identification in the Gc of CCHFV [7,21,22]. Although several immunodominant region have been mapped on CCHFV-Gc, to our knowledge, no minimal motifs have been previously identified.

In this study, we mapped minimal epitopes on CCHFV-Gc using pAbs against CCHFV-Gc. All the epitopes could be recognized by the antiserum of sheep infected with CCHFV. We also analyzed the conservation of each epitope among homologous CCHFV Gc fragment, and their positions in the predicted three-dimensional (3D) structure of Gc fragment. The results of this study would help us to understand more about epitope distribution on CCHFV-Gc, and would provide solid foundation for the design and development of a preventative CCHFV multi-epitope peptide vaccine and detection antigen.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Research Ethics Committee (Animal Ethics Committee of Xinjiang University). All procedures were conducted in accordance with the policies and regulations of Experimental Animals of China [20].

2.2. Plasmids, antibodies and strains

The recombinant (r-) plasmid pET-28a-*Gc* (aa 1–558) were previously constructed and saved by our research group [23]. The prokaryotic expression plasmid pXXGST-1 was donated by Professor Wanxiang Xu from Shanghai Institute of Planned Parenthood Research (Shanghai, China). Rabbit pAbs against CCHFV-Gc was obtained from Professor Fei Deng of Chinese Academy of Sciences, Wuhan Institute of Virology. New Zealand rabbits were injected intramuscularly with 0.5 mg of purified recombination Gc segment and immunized at two-week intervals according to the conventional animal immune method [20]. After the third immunization for two weeks, rabbit antiserum was separated and stored at -80℃ until use. The sheep sera used in the study were provided by Professor Yujiang Zhang of Xinjiang Centers for Disease Control and Prevention (XJCDC). Healthy and CCHFV-infected sheep sera were previously identified using an indirect immunofluorescent assay (IFA) and reverse transcription polymerase chain reactions (RT-PCRs) [19]. *Escherichia coli* (*E. coli*) BL21 (DE3) competent cells were used to express 16/8mer peptides fused with a truncated GST188 protein (initial 188 aa of glutathione S-transferase) [19,20]. Goat anti-rabbit, Goat anti-mouse and mouse anti-goat IgG conjugated to horseradish peroxidase (HRP) were purchased from Beijing TransGen Biotech, Co., Ltd. (China).

2.3. Reagents and materials

Kpn I, *Xho* I, *Bam*H I, *Sal* I, and DNA ligase (Takara Co., Ltd. Dalian, China), *E. coli* BL21 (DE3) competent cells (Novagen, Inc., Madison, USA), QIA quick Gel Extraction Kit (QIAGEN, Duesseldorf, Germany), unstained or prestained molecular weight markers (Thermo Fisher Science, Waltham, MA, USA), nitrocellulose membranes (Whatman GmbH, Dossel, Germany), enhanced chemiluminescence (ECL) plus western blotting detection kit (GE Healthcare, Buckinghamshire, UK) were obtained. Keyhole limpet hemocyanin (KLH) conjugated synthetic peptides were synthesized on the APEX396 synthesis instrument (Hyosung Biotechnology Co., Ltd, Shanghai, China). The purity of the synthesized peptides was above 95% by HPLC and ESI-MS analysis. Other general chemicals were obtained from Shanghai Sangon Co., Ltd. (Shanghai, China).

2.4. Construction of recombinant plasmids of the truncated segments

According to bioinformatics analysis, CCHFV YL04057 Gc fragment was divided into five truncated segments (Fig. 1). *GcI* (aa 1–196), *GcII* (aa 180–300), *GcIII* (aa 197–436), Gc*IV* (aa 407–530), and *GcV* (aa 437–558) were designed using previously constructed pET-28a-*Gc* as the template and amplified by PCR (Table 1). To construct the prokaryotic expression plasmid of five truncated Gc segments, the

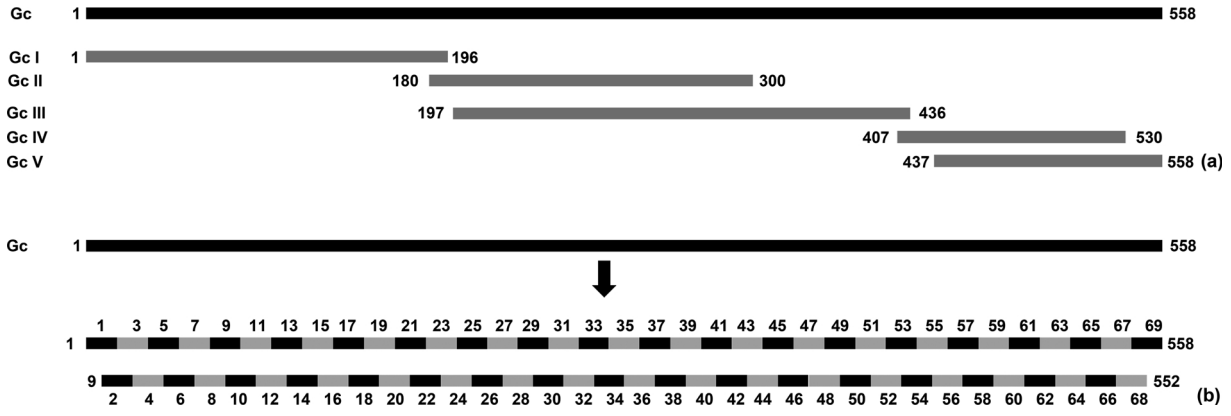

Fig. 1. Schematic of epitope mapping strategy. (a) The black band indicates the full length of the Gc fragment, the gray bands indicate the Gc*I-V* segments. (b) Schematic of epitope mapping strategy involving 69 overlapping 16mer peptides spanning Gc.

Table 1
Primers used for truncated proteins GcI-V.

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
GcI	ATACCATGGTCAGGAAGCCACTCTTCCTGG	ATCTCGAGTTCTGTCTTTATATATTC
GcII	ATACCATGGATTTTACTGACTACATGCTG	ATCTCGAGTGAGTGTCCATTAAC
GcIII	TATCCATGGAAGCTATAGTGTGTGTCG	TACTCGAGTTCATTTACATGGATTC
GcIV	TATCCATGGTGTCAGGTTTGAAAATTGC	TACTCGAGACAAGTGTCATTTTGC
GcV	TATCCATGGAACCAGATGAATTTACAG	ATCTCGAGATTTCCAAAGATGCCAC

Note: underlined regions represent *Kpn* I and *Xho* I restriction sites, respectively.

amplified segments were digested with *Kpn* I and *Xho* I and inserted into the prokaryotic expression vector pET-28a or pET-32a, respectively.

2.5. Expression and antigenicity identification of the truncated segments

The five r-plasmids identified correctly by double enzyme digestion and sequencing were transformed into *E. coli* BL21 (DE3) competent cells, with each r- clone was cultivated in 3 mL Luria-Bertani (LB) medium containing 50 μg/mL ampicillin or kanamycin at 220 rpm/min overnight. The following day, 20 μL of cell suspension was added to 2 mL of fresh LB medium, grown at 37 ℃ for 4 h to increase the bacterial density until reaching an optical density at 600 nm (OD_{600}) of 0.5-0.7, cells were induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside to express protein, which was subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by western blot to detect the antigenicity of the r-protein.

2.6. Mapping strategy and biosynthesis of overlapping 16/8mer peptides

To complete linear BCEs mapping of CCHFV Gc, we designed 16mer peptides containing an overlap of 8-aa residues that covered the full-length of the Gc segment for the first round of antigenic peptide mapping (Fig. 1). Additionally, a set of 8mer peptides with overlapping 7-aa residues for coverage of each reactive 16mer peptide sequence for the second round of precise BCE-motif identification were designed [13]. A total of 69 16mer peptides were designed, all of which contained an overlap of 8-aa residues between two adjacent peptides. For fine-epitope-motif mapping, 70 8mer peptides with an overlap of 7-aa residues were expressed according to the results of 16mer antigenic peptide mapping.

2.7. Construction of recombinant plasmids and induced their expression

All corresponding plus and minus stands of gene fragments that encoding the 16/8mer peptides harbored cohesive end nucleotides containing *Bam*H I and TAA-*Sal* I sites at the 5′- and 3′- ends were synthesized by SBS Genetech Co., Ltd (Shanghai, China), respectively. After ligation of the annealed DNA fragment into the pXXGST-1 plasmid digested with *Bam*H I and *Sal* I, the mixture was transformed into *E. coli* BL21 (DE3) competent cells and ultimately screened for the r-clone by SDS-PAGE using total proteins from each induced clone to determine the presence of the specific 16/8mer peptide. Prior to transformation, sequencing was performed on the inserted DNA fragment encoding each 16/8mer peptide to ensure the accuracy of all synthesized DNA sequences [13].

The 16/8mer peptides fused to the truncated GST188 protein were expressed in *E. coli* BL21 (DE3) cells, and the selected r-clones were cultivated in 2 mL LB medium (50 μg/mL ampicillin) at 37 ℃ with shaking at 220 rpm/min overnight. The following day, 20 μL of the culture was transferred to 2 mL of fresh LB medium and grown at 30 ℃ until reaching an optical density at 600 nm (OD_{600}) of 0.6-0.8, and then grown at 42 ℃ for 4 h to induce protein expression. SDS-PAGE (10%) analysis was performed to screen for positive clones, with positive strains subsequently sequenced for verification. The bacterial pellets containing the short 16/8mer peptide fusion proteins were stored at − 20 ℃.

2.8. SDS-PAGE and western blot

The expressed r-proteins were boiled in 200 μL of 5× loading buffer for 15 min, and the proteins were detected by SDS-PAGE under reducing conditions using a 10% gel. Gels were either stained with Coomassie brilliant blue R-250 for analyzing the bands corresponding to the target protein. The other gel was used for western blotting by electrotransferring the proteins onto a 0.2 μm nitrocellulose (NC) membrane. Regarding the specific antigen-antibody reaction, the NC membrane was blocked with 5% (w/v) non-fat milk powder in TBS-T, treated with the pAbs (1:500 dilution for r-protein; 1:1000 dilution for 16/8 mer peptides) or sheep sera (1:100 dilution) as the primary antibody, and then a specific antigen-antibody reaction on the membrane was visualized using goat anti-rabbit or mouse anti-goat IgG conjugated to HRP (1:5000 dilution) as a secondary antibody. Finally, the blot was performed using the ECL plus western blotting detection reagent according to the manufacturer's instructions, and it was then imaged by GE-Image Quant LAS 4000 (GE Healthcare, Buckinghamshire, UK).

2.9. Detection immune-reactivity of antigenic epitopes by Dot-ELISA

According to Dot-ELISA method [24], NC membrane was cut into strips and soaked in PBS (0.01 M pH 7.4) for 1 h then allowed to air dry at room temperature. Synthetic epitope peptides, rGc (as a positive control) and KLH (as a negative control) were ground and dispersed in the elution buffer (100 μL). All the dispersions were centrifuged at 12,000 rpm/min for 5 min, to deposit the insoluble fraction.1 μL solution was then deposited on the membrane. After drying, the strips were blocked with 5% skimmed milk for 2 h at 37 ℃. After three washes with PBS-T, the strips were incubated with positive sheep serum collected from Guertu county in Xinjiang with a confirmed history of CCHFV infection for verify the immune-reactivity of the epitopes for 1 h at 37 ℃. Rabbit anti-goat IgG antibody conjugated to HRP (1:2000 dilution) was used as the secondary antibody at 37 ℃ for 1 h, followed by colour development with DAB. The reactions were stopped by rinsing the membrane with distilled water.

2.10. Sequence conservation analysis and 3D modeling

To assess the conservation of each identified epitope among CCHFV homologous proteins, ten Gc aa sequences from different countries were obtained from GenBank datebase based on the phylogenetic tree of CCHFV strains. The aa sequences of the Gc segments from the YL04057 strain (GenBank code: ACM78470.1) and other CCHFV-Gc homologous proteins were aligned using the ClustalW program (http://www.ebi.ac.uk/services) [25] and visualized using Gene doc [26].

The location of experimentally identified linear BCEs in the tertiary structure of the CCHFV Gc fragment was analyzed by PyMOL™ software (https://pymol.org/2/). The prediction of secondary structure was based on Garnier and Robson [27], Chou and Fasman [28]. The hydrophilic scheme, flexible regimen, surface accessibility regimen and antigenicity index were analyzed and predicted using the methods of Kyte-Doolittle [29], Karplus-Schulz [30], Emini [31] and Jameson-Wolf

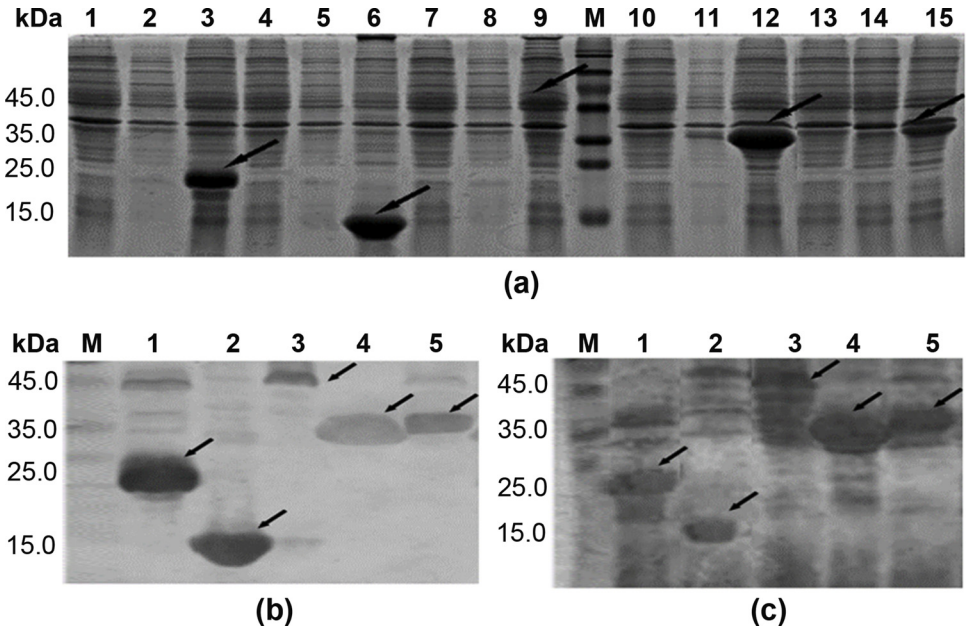

Fig. 2. Prokaryotic expression of recombinant truncated Gc segments and immunoblot analysis of the five recombinant Gc truncated segments. (a) Recombinant truncated Gc segments were expressed as His-tagged fusion proteins in *E. coli* BL21 (DE3) cells and subjected to SDS-PAGE analysis. Lanes 1, 4, 7, 10 and 13: uninduced cells harboring the pET-28a-*GcI-V* plasmids; Lanes 2, 5, 8, 11, and 14: supernatant of lysed cells harboring pET28a-G*cI-V*; Lanes 3, 6, 9, 12, and 15: The precipitate from cells harboring pET28a-G*cI-V*. (b) Western blot of rGcI-V using goat anti-His mouse monoclonal antibody. (c) Western blot of rGcI-V using rabbit pAbs against r-Gc. The arrows represent the five expressed target segments on the gel and the reactive segments in the western blot analysis.

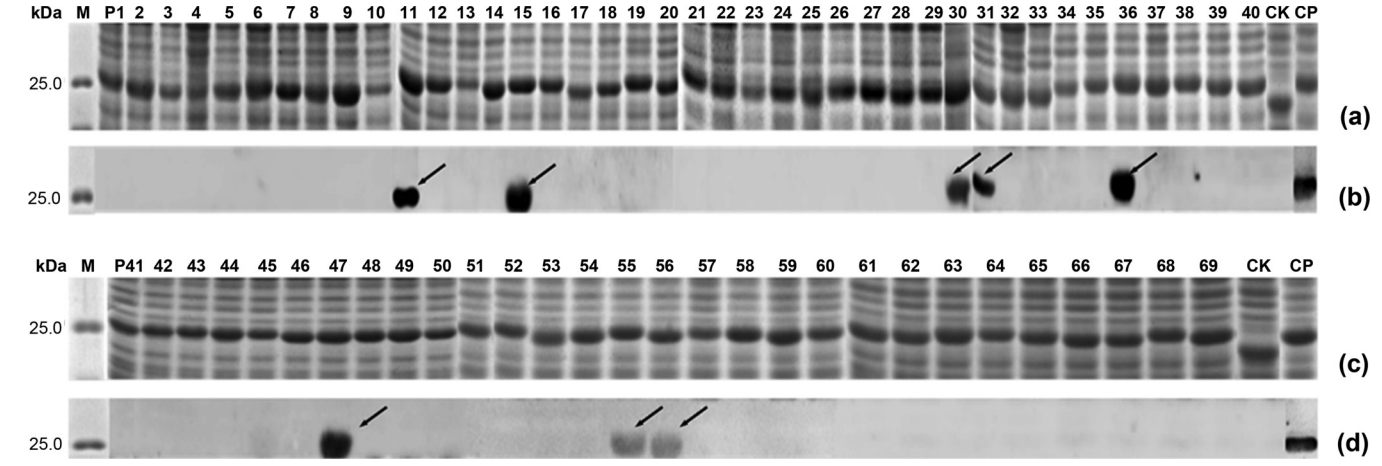

Fig. 3. SDS-PAGE and western blot analyses of 16mer peptides derived from the CCHFV Gc fragment. Arrows represent the peptides with a positive antigen-antibody reaction in western blotting analysis. SDS-PAGE (a, c) and western blot (b, d) analyse of 69 GST-fused 16mer peptides. Lanes 1–69, GST-fused 16mer peptides (P1-P69). CK, negative control (GST188 protein expressed by pXXGST-1); CP, positive control of expressed 16mer peptide.

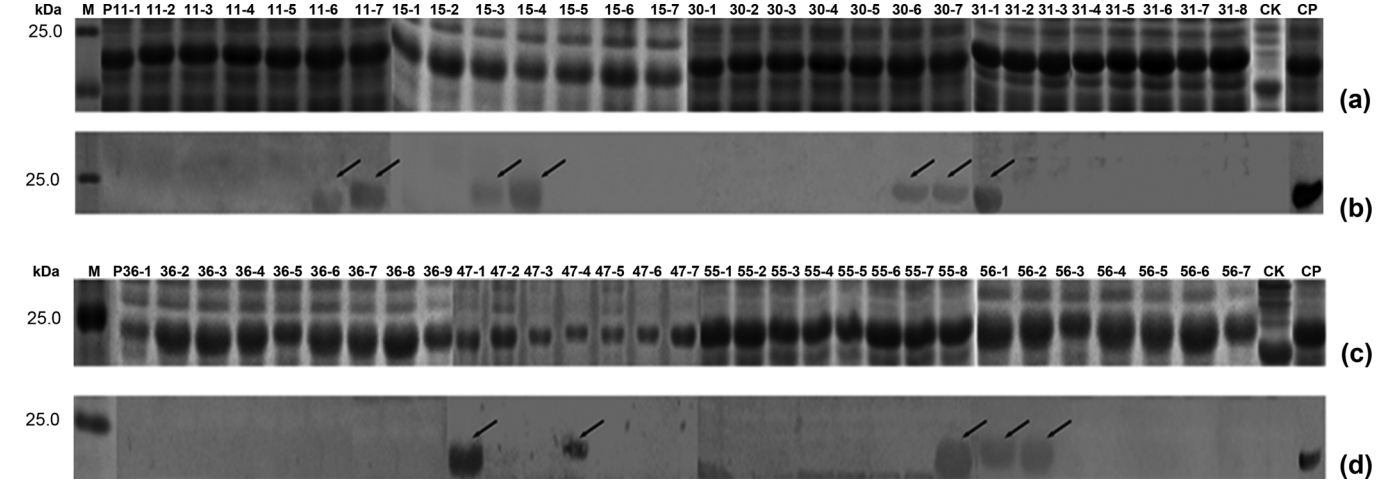

Fig. 4. SDS-PAGE and western blot determination of minimal motifs of the identified BCEs. Arrows represent the corresponding positive antigenic-peptides according to western blot analysis. SDS-PAGE (a, c) and western blot (b, d) analyses of 70 GST-fused 8mer peptides. CK, negative control (GST188 protein expressed by pXXGST-1). CP, positive control (8 of 16mer peptides were identified as positive by pAbs).

Peptide items	Amino acids	Position in Gc	Peptide items	Amino acids	Position in Gc
P1	RKPLFLDSIAKGKKSL	1-16	P36	DFQVYHVGNLLRGDKV—AP-4	281-296
P2	IAKGKKSLLNSTSLET	9-24	P36-1	DFQVYHVG	281-288
P3	LNSTSLETSLSIEAPW	17-32	P36-2	FQVYHVGN	282-289
P4	SLSIEAPWGAINVQST	25-40	P36-3	QVYHVGNL	283-290
P5	GAINVQSTFKPTVSAA	33-48	P36-4	VYHVGNLL	284-291
P6	FKPTVSAANIALSWSS	41-56	P36-5	YHVGNLLR	285-292
P7	NIALSWSSVEHRGNKI	49-64	P36-6	HVGNLLRG	286-293
P8	VEHRGNKILVSGRSES	57-72	P36-7	VGNLLRGD	287-294
P9	LVSGRSESIMKLEERT	65-80	P36-8	GNLLRGDK	288-295
P10	IMKLEERTGVSWNLGV	73-88	P36-9	NLLRGDKV	289-296
P11	GVSWNLGVEDASESKT	81-96	P37	NLLRGDKVNGHSIHKI	289-304
P11-1	VSWNLGVE	82-89	P38	NGHSIHKIEPHFNTSW	297-312
P11-2	SWNLGVED	83-90	P39	EPHFNTSWMSWDGCDL	305-320
P11-3	WNLGVEDA	84-91	P40	MSWDGCDLDYFCNMGD	313-328
P11-4	NLGVEDAS	85-92	P41	DYFCNMGDWPSCTYTG	321-336
P11-5	LGVEDASE	86-93	P42	WPSCTYTGVTQHNRAA	329-344
P11-6	GVEDASES E1	87-94	P43	VTQHNRAAFINMLNIE	337-352
P11-7	VEDASESK E1	88-95	P44	FINMLNIETDYTKTFH	345-360
			P45	TDYTKTFHFHSKRVTA	353-368
P12	EDASESKTLTVSVMDL	89-104	P46	FHSKRVTAHGDTPQLD	361-376
P13	LTVSVMDLSQMYSPVF	97-112			
P14	SQMYSPVFEYLSGDRQ	105-120	P47	HGDTPQLDLKARPAYG	369-384
			P47-1	GDTPQLDL—E5a	370-377
P15	EYLSGDRQVEEWPKAT	113-128	P47-2	DTPQLDLK	371-378
P15-1	YLSGDRQV	114-121	P47-3	TPQLDLKA	372-379
P15-2	LSGDRQVE	115-122	P47-4	PQLDLKAR—E5b	373-380
P15-3	SGDRQVEE E2	116-123	P47-5	QLDLKARP	374-381
P15-4	GDRQVEEW E2	117-124	P47-6	LDLKARPA	375-382
P15-5	DRQVEEWP	118-125	P47-7	DLKARPAY	376-383
P15-6	RQVEEWPK	119-126			
P15-7	QVEEWPKA	120-127	P48	LKARPAYGAGEVTVLV	377-392
			P49	AGEVTVLVEVADLELH	385-400
P16	VEEWPKATCTGDCPER	121-136	P50	EVADLELHTKKLEVSG	393-408
P17	CTGDCPERCGCTSSTC	129-144	P51	TKKLEVSGLKIASLTC	401-416
P18	CGCTSSTCLHKEWPHS	137-152	P52	LKIASLTCSGCYACSS	409-424
P19	LHKEWPHSRNWRCSPT	145-160	P53	SGCYACSSGISCKVRI	417-432
P20	RNWRCSPTWCWGVGTG	153-168	P54	GISCKVRIHVNEPDEF	425-440
P21	WCWGVGTGCTCCGLDV	161-176			
P22	CTCCGLDVKDFFTDYM	169-184	P55	HVNEPDEFTVHVRSSD	433-448
P23	KDFFTDYMLVKWKVEY	177-192	P55-1	HVNEPDEF	433-440
P24	LVKWKVEYIKTEAIVC	185-200	P55-2	VNEPDEFT	434-441
P25	IKTEAIVCVELTSQER	193-208	P55-3	NEPDEFTV	435-442
P26	VELTSQERQCSLIEAG	201-216	P55-4	EPDEFTVH	436-443
P27	QCSLIEAGTRFNLGPV	209-224	P55-5	PDEFTVHV	437-444
P28	TRFNLGPVTITLSEPR	217-232	P55-6	DEFTVHVR	438-445
P29	TITLSEPRNVQQRLPP	225-240	P55-7	EFTVHVRS	439-446
			P55-8	FTVHVRSS	440-447
P30	NVQQRLPPEIVTLHPK	233-248			
P30-1	VQQRLPPE	234-241	P56	TVHVRSSDPDVVAAGS	441-456
P30-2	QQRLPPEI	235-242	P56-1	TVHVRSSD E6	441-448
P30-3	QRLPPEIV	236-243	P56-2	VHVRSSDP E6	442-449
P30-4	RLPPEIVT	237-244	P56-3	HVRSSDPD E6	443-450
P30-5	LPPEIVTL	238-245	P56-4	VRSSDPDV	444-451
P30-6	PPEIVTLH E3	239-246	P56-5	RSSDPDVV	445-452
P30-7	PEIVTLHP E3	240-247	P56-6	SSDPDVVA	446-453
			P56-7	SDPDVVAA	447-454
P31	EIVTLHPKIEEGFFDL	241-256			
P31-1	EIVTLHPK E3	242-249	P57	PDVVAAGSSLMARKIE	449-464
P31-2	IVTLHPKI	243-250	P58	SLMARKIEFGADSTFK	457-472
P31-3	VTLHPKIE	244-251	P59	FGADSTFKAFSSMPKD	465-480
P31-4	TLHPKIEE	245-252	P60	AFSSMPKDSLCFYIVE	473-488
P31-5	LHPKIEEG	246-253	P61	SLCFYIVEKDYCSSCT	481-496
P31-6	HPKIEEGF	247-254	P62	KDYCSSCTDDDTQKCV	489-504
P31-7	PKIEEGFF	248-255	P63	DDDTQKCVNTKLDHPQ	497-512
P31-8	KIEEGFFD	249-256	P64	NTKLDHPQSILIEHKG	505-520
			P65	SILIEHKGTIIGKQND	513-528
P32	IEEGFFDLMHVQKILS	249-264	P66	TIIGKQNDTCSTKTSC	521-536
P33	MHVQKILSASTVCKLQ	257-272	P67	TCSTKTSCWLESVKGF	529-544
P34	ASTVCKLQSCTHGVPG	265-280	P68	WLESVKGFFYGLKNML	537-552
P35	SCTHGVPGDFQVYHVG	273-288	P69	FYGLKNMLSGIFGNGG	545-560

Fig. 5. The bio-synthetic overlapping peptides derived from IgG-reactive peptides of Gc^{1-558}. The yellow highlighting represents the common sequences among immunedominant peptides that react with pAbs according to western blotting analysis.

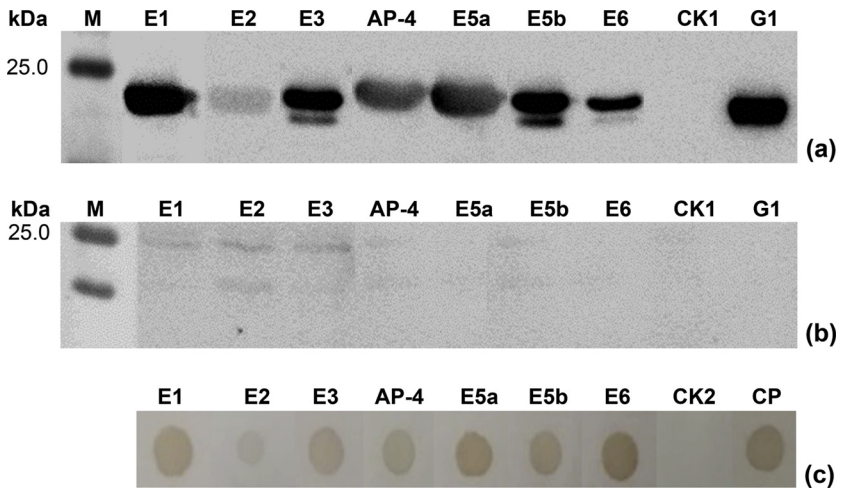

Fig. 6. Western blot and Dot-ELISA of seven peptides containing identified epitopes performed using sheep sera. (a) A positive serum sample from a sheep with a confirmed history of CCHFV infection. (b) A negative serum sample from healthy sheep with no history of CCHFV infection. CK1, negative control (GST188 protein expressed by pXXGST-1); G1, using a 16mer peptide recognized by sheep positive serum as positive control. (c) Immuno-reactivity of synthetic peptide with antibody positive sheep serum naturally infected with CCHFV. CK2, negative control (KLH); CP, positive control (rGc).

[32]. Based on the results obtained from these analyses, peptides with good hydrophilicity, high accessibility, high flexibility and strong antigenicity were selected as epitope candidates.

3. Results

3.1. Expression of truncated segments and antigenic analysis of the truncated Gc segments

The recombinant plasmids pET28a-*GcI*, pET28a-*GcII,* pET32a-*GcIII*, pET32a- *GcIV* and pET32a-*GcV* were successfully constructed by sequencing. SDS-PAGE analysis confirmed the presence of proteins at 23 kDa, 14 kDa, 45 kDa, 33 kDa, and 35 kDa representing products size, respectively. Western blot analysis of the five truncated CCHFV Gc segments using goat anti-mouse antibody and anti-Gc rabbit pAbs showed that all were correctly expressed and could be recognized, indicating that the five fragments displayed good antigenicity and contained Gc linear epitopes (Fig. 2).

3.2. Identification of 16mer peptides containing linear BCEs

To determine the existence of BCEs in the Gc fragment, the five truncated segments were converted into 16mer biosynthetic peptides, respectively. The 69 overlapping 16mer peptides were fused with GST188 and expressed in *E. coli*, resulting in fusions of 23 kDa according to SDS-PAGE analysis (Fig. 3a and c). Among these peptides, eight positive 16mer peptides including the P11 (GVSWNLGVEDASE-SKT), P15 (EYLSGDRQVEEWPKAT), P30 (NVQQRLPPEI VTLHPK), P31 (EIVTLHPKIEEGFFDL), P36 (DFQVYHVGNLLRGDKV), P47 (H GDTP-QLDLKARPAYG), P55 (HVNEPDEFTVHVRSSD) and P56 (TVHVRSS-DPD VVAAGS) (Fig. 3b and d) were found to specifically bind to pAbs and were identified as potential linear BCEs.

3.3. Identification of the minimal motifs for the detected BCEs

To map the epitopes on the Gc segment, we constructed and expressed for sets of overlapping 8mer peptides after mapped reactive 16mer-peptides, which were used in the second round of fine epitope mapping. The overlapping 16/8mer peptide sequences and their corresponding locations in the Gc fragment are shown in Fig. 5. SDS-PAGE results showed that all GST188-tagged 8mer peptides were correctly expressed in *E. coli* (Fig. 4a and c), and western blot confirmed that two 8mer-peptides of P11-6 and P11-7 in P11 were able to react specifically with rabbit pAbs, indicating that the minimum motif of named E1 epitope is VEDASES because of the common sequence present in them. Similarly, of expressed overlapping 8mer peptides from other seven reactive 16mer peptides, P15-3 and P15-4 in P15, P30-6, P30-7 and P31-1 in P30 and P31, P47-1, P47-4 in P47, and P55-8, P56-1 and P56-2 in P55 and P56 were found to be able to react with rabbit pAbs as well (Fig. 4). Interestingly, overlapping 8mer peptides P36-1 to P36-9 in one positive 16 mer peptide P36 did not show a positive reaction indicated that its minimal epitope motif may contain longer antigenic peptides (AP). Therefore, this result indicated that there were identified six BCEs and one antigenic peptide named E1 (VEDASES), E2 (GDRQVEE), E3 (EIVTLHP), AP-4 (DFQVYHVGNLRGDKV), E5a (GDTPQLDL), E5b (PQLDLKAR) and E6 (HVRSSD), respectively.

3.4. Reactivity of the identified epitope motifs with anti-CCHFV sera

To determine whether the BCEs specifically identified by pAbs could also recognized by other host species, six 8mer peptides and one 16mer peptides containing pAbs-identified minimal motifs were subjected to western blot using healthy or CCHFV-infected sheep sera [33]. All the selected seven peptides were recognized by the serum of sheep with a confirmed history of CCHFV infection while none reacted with the CCHFV antibody-negative sheep serum (Fig. 6a and b). Dot-ELISA results also showed that all the identified epitopes could bind to the antibody positive sheep serum from the natural infection CCHFV (Fig. 6c). Of the seven peptides, the five 8mer peptides P11-7 (containing E1), P30-6 (containing E3), P47-1 (containing E5a), P47-4 (containing E5b), P56-2 (containing E6) and one 16mer peptide P36 (containing AP-4) showed the strongest reaction with CCHFV-infected sheep serum; Meanwhile, the 8mer peptide P15-3 (containing E2) displayed the weaker reaction (Fig. 6a and c).

3.5. Sequence conservative analysis and 3D modeling

To analyze the conservation of each BCEs, multiple sequence alignment was conducted using the aa residues Gc $^{1\text{-}558}$ from the Chinese CCHFV strain YL04057 (GenBank accession number: ACM78470.1) and 10 other strains from different countries, such as Chinese strain: 79121 (GenBank accession number: AB069673), BA66019 (GenBank accession number: AF350448), BA88166 (GenBank accession number: AF338470) Pakistan strain: Matin (GenBank accession number: AF467769); South African strain: SPU415/85 (GenBank accession number: DQ211635); Nigerian strain: IbAr10200 (GenBank accession number: AF467768); Uzbekistan strain: Hodzha (GenBank accession number: AY223476); Russian strain: ROS/HUVLV-100 (Gen Bank accession number: DQ206448); Turkish strain: Turkey-Kelkit06 (GenBank accession number: GQ337054) and Yugoslavia strain: Kosova Hoti (GenBank accession number: EU037902). The results indicated that E1, E5a, and E5b displayed 100% homology, whereas E2, E3, AP-4, and E6 showed differences in individual aa residues, resulting in 98.6%, 88.3%, 79.4% and 75% sequence similarities, respectively (Fig. 7).

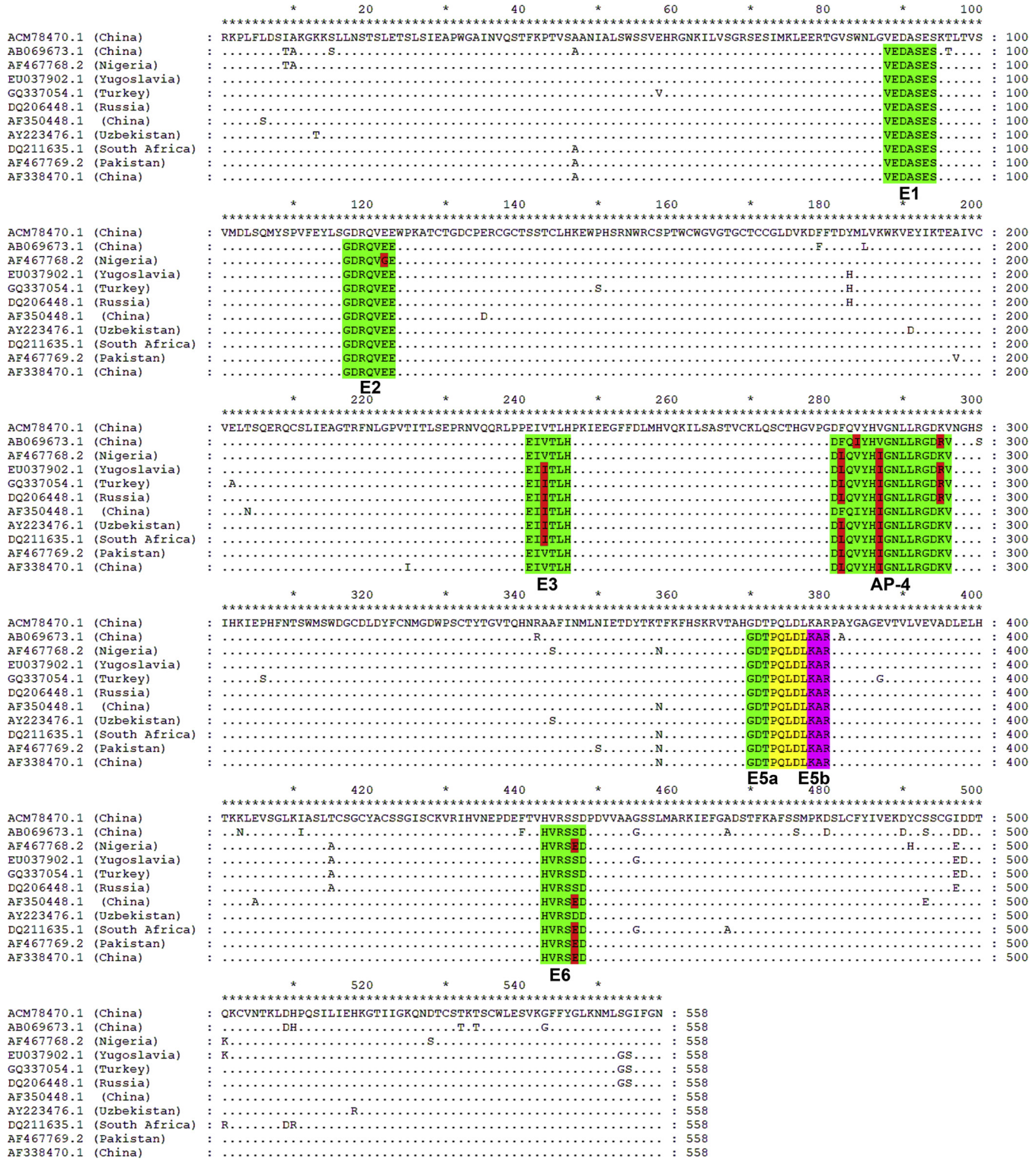

Fig. 7. Amino acid sequence comparison of the $Gc^{1–558}$ fragment from the YL04057 strain and other CCHFV strains using the ClustalW program. The GenBank codes are shown on the left. The seven minimal epitopes (E1, E2, E3, AP-4, E5a, E5b, and E6) recognized by the rabbit pAbs are highlighted and the variable aa residues within the minimal motif epitopes are highlighted in red. Dots (.) indicate identical aa residues within all 10 strains. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Except for AP-4 and E6, the remaining five epitopes displayed > 85% sequence homology.

PyMOL™ software [34] was used to simulate the 3D structure of CCHFV YL04057 Gc to locate all the mapped epitopes. Labeled with different colors, result shows that the identification of all BCEs was located on the surface of the Gc 3D space structure (Fig. 8b), which facilitates antibody-specific binding.

The secondary structure of the $Gc^{1–558}$ was predicted by DNASTAR-Protean software (DNASTAR Inc, Madison, WI, USA). The secondary structure prediction result showed that the identified epitopes form part of a beta-sheet and alpha-helices regions (Fig. 8a). Furthermore, the identified epitopes were predicted to be located on the surface of

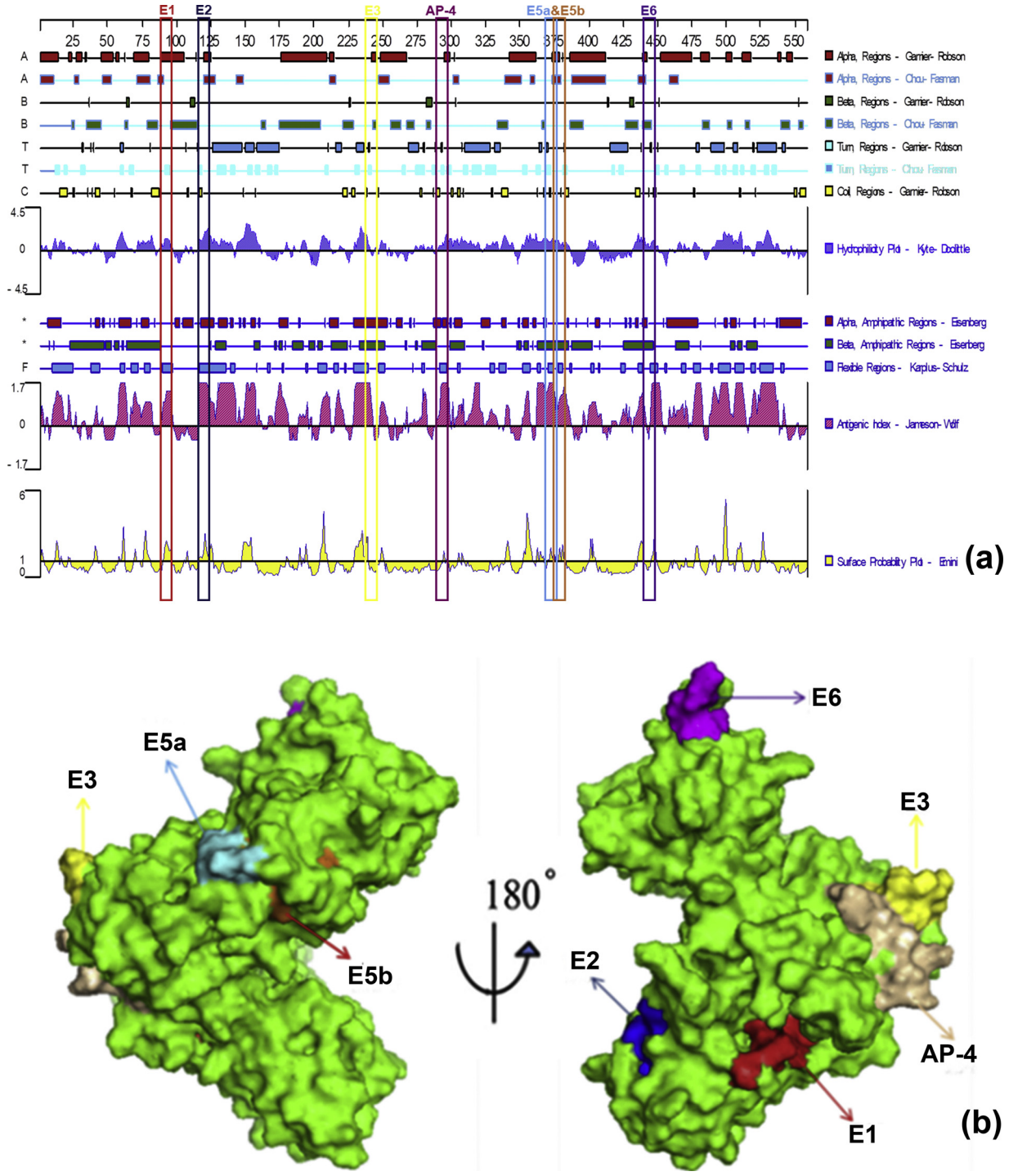

Fig. 8. Prediction of Gc protein secondary structure and localization on 3D structure of mapped BCEs on CCHFV-Gc. (a) Epitope prediction for aa residues 1–558 of Gc from YL04057 strain using DNAStar-Protean software. The secondary structure of E1 (red), E2 (dark blue), E3 (yellow), AP-4 (light powder), E5a (light blue), E5b (orange), and E6 (purple) on secondary structure are shown. The flexibility plot, hydrophilicity, surface probability, and antigenicity index for Gc 1–558 segment were taken into consideration. (b) The BCE surfaces of six BCEs and one AP on molecular surface are shown. The figures were generated using the PyMOL™ molecular graphics system.

CCHFV Gc, with a high antigenic index and hydrophilicity, suggesting that the identified epitope was likely to be an important B-cell epitope on Gc fragment of CCHFV YL04057 (Fig. 8a). 3D models and protein secondary structure predictions indicated that the predicted results were consistent with the identified epitopes, and the identified BCEs were all located in the hydrophilic region.

4. Discussion

In 1965, the first case of CCHF in China was found in Bachu County in Xinjiang [33]. This was followed by sporadic outbreaks, with the mortality rate in Bachu County in the spring of 2001 and associated with the disease reaching 5.88% (3/51) [35,36]. Subsequently, potential therapeutic strategies have not been confirmed by clinical trials, and although ribavirin have shown different degrees of efficacy in

sporadic outbreak, there remains no effective diagnostic treatment or vaccine [2].

Glycoprotein Gc is a major antigen of CCHFV, plays a key role in the fusion of virus to host cells and can mediate viral entry into a variety of human and animal cell to induce the production of neutralizing antibodies [37,38]. As the only virally encoded membrane proteins, Gn and Gc must interact with cell surface receptors, mediate the entry of virus into cells, and serve as targets for neutralizing antibodies [39,40]. Bertolotti et al. developed a panel of MAbs to assist in studies on CCHFV glycoprotein biology and to begin characterizing the antigenic structures of Gn and Gc [6]. The large majority of MAbs bound to conformation-dependent epitopes in Gn or Gc. A number of MAbs against Gc, but not against Gn, were able to neutralize virus infection of SW-13 cells in vitro, suggesting that Gc plays an important role in virus entry, and this is by far the clearest indication that Gc not only binds target cells but also mediates virus entry [6,7]. Identification of BCEs for the CCHFV Gc protein has been limited [22,41,42]. The identification of these antibody binding sites provides new targets for the design and development of vaccines and diagnostic reagents [19,36]. Therefore, investigation of the antigenicity of the CCHFV Gc fragment and subsequent identification of the associated antigenic regions are of great significance for the development of vaccines or testing reagents. However, up to date, there are no reports on epitope mapping of CCHFV Gc fragment and their fine localization.

In this study, to carry out a comprehensive linear epitope scan of CCHFV-Gc, the modified epitopes of Gc fragment was identified using a modified BSP method. First, we synthesized 69 overlapping 16mer peptides and 70 overlapping 8mer peptides, seven BCEs on Gc (aa 1–558) protein from CCHFV YL04057 stain were obtained: E1 (^{88}VEDASES^{94}), E2 (^{117}GDRQVEE^{123}), E3 (^{241}EIVTLH^{246}), AP-4 (^{281}DFQVYHVG NLLRGDKV^{296}), E5a (^{370}GDTPQ LDL^{377}), E5b (^{373}PQLDLKAR^{380}) and E6 (^{443}HVR SSD^{448}). Second, to further validate the antigenicity of mapped each BCE, western blot and Dot-ELISA were carried out using antibody-positive sheep serum with confirmed CCHFV infection, and the result revealed that all the BCEs could be specifically recognized by the sheep serum, showing the feasibility and meaning of our identification approach. All motifs were exposed on the surface of the 3D structure, in the helix-angle-helix region (Fig. 8b), indicating that they could easily bind to antibodies. The epitopes located on the surface of the target protein play an important role in the future development of drugs that interact with target antigens. Interestingly, our results showed that there is a certain difference in antigenicity between each epitope. Among the identified epitopes, only the 8mer peptide containing E2 epitope showed weaker antigenicity although it located on the surface of the Gc fragment, which is located in the hydrophilic region (Fig. 8a). It was suggested that epitope of E2 has a loosely coiled structure (Fig. 8a), which is greatly affected by the side chain interactions, and therefore has a certain influence on its-antigenicity [43].

The sequences of 10 strains of CCHFV Gc from different countries were analyzed. The results showed that three epitopes, E1, E5a, and E5b were 100% conserved among the 10 CCHFV strains, while the epitopes of E2, E3, and E6 displayed differences in only a single aa difference. The minimal antigenic epitopes E1, E2, E3, E5a, E5b and E6 identified in this study were highly conserved, suggesting their potential efficacy as universal epitopes for CCHF diagnosis in the future and these epitopes can be used for animal epidemiological investigation in the epidemic area of CCHFV. However, the antigenicity of these epitopes identified in this study was identified using rabbit polyclonal serum and should further verified using human serum derived from CCHFV infected individuals. Furthermore, combinatorial use of dominant multi-epitope peptide as an antigen could potentially be utilized for comprehensive analysis in order to enhance the understanding of CCHFV prevalence.

In conclusion, six fine epitope motifs and one antigenic peptide were obtained, which provided new data for the development of a CCHFV multi-epitope detection kit and a multi-epitope peptide vaccine. These results would promote elucidation of the pathogenic mechanism of CCHFV and it could lay the foundation for the design and development of a CCHFV multi-epitope peptide vaccine and detection antigen.

Declaration of Competing Interest

Nothing to claim.

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