AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

PATHOGENESIS AND IMMUNITY

Cross-Reactive Anti-Nucleocapsid Protein Immunity against Crimean-Congo Hemorrhagic Fever Virus and Hazara Virus in Multiple Species

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ABSTRACT The World Health Organization estimates that there may be three billion people at risk of infection by Crimean-Congo hemorrhagic fever virus (CCHFV), a highly lethal emerging *Orthonairovirus* carried by ticks. On the other hand, the closely related Hazara virus (HAZV), a member of the same serogroup, has not been reported as a pathogen for humans. Given the structural and phylogenetic similarities between these two viruses, we evaluated the immunological similarities of the nucleocapsid protein (NP) of these two viruses in multiple species. Strong antigenic similarities were demonstrated in anti-NP humoral immune responses against HAZV and CCHFV in multiple species using convalescent-phase human CCHF sera, rabbit and mouse polyclonal antiserum raised against CCHFV, and mouse polyclonal antiserum against CCHFV-NP in enzyme immunoassays. We also report a convincing cross-reactivity between NPs in Western blots using HAZV-infected cell lysate as antigen and inactivated CCHFV- and CCHFV-NP-immunized mouse sera. These results suggest that NPs of HAZV and CCHFV share significant similarities in humoral responses across species and underline the potential utility of HAZV as a surrogate model for CCHFV.

IMPORTANCE Crimean-Congo hemorrhagic fever virus (CCHFV) and Hazara virus (HAZV), members of the *Nairoviridae* family, are transmitted to mammals by tick bites. CCHFV is considered a severe threat to public health and causes hemorrhagic diseases with a high mortality rate, and there are neither preventative nor therapeutic medications against CCHFV disease. HAZV, on the other hand, is not a pathogen to humans and can be studied under biosafety level 2 (BSL2) conditions. The antigenic relationship between these viruses is of interest for vaccines and for preventative investigations. Here, we demonstrate cross-reactivity in anti-nucleocapsid protein (NP) humoral immune response between NPs of HAZV and CCHFV in multiple species. These results underline the utility of HAZV as a surrogate model to study CCHFV infection.

KEYWORDS HAZV, CCHFV, nucleocapsid protein, humoral immunity, antigenic similarity, humoral immune response, Crimean-Congo hemorrhagic fever virus, HAZV

Hazara virus (HAZV) is an agent that was isolated from ticks in the Hazara region of West Pakistan in 1964 (1, 2). HAZV is classified in the *Nairoviridae* family, along with the Crimean-Congo hemorrhagic fever virus (CCHFV), and has not been classified as a human pathogen. However, CCHFV causes severe hemorrhagic fever in humans, and the mortality rates reach up to 30% (3). CCHFV has a wide distribution throughout Africa, Asia, the Middle East, and Eastern Europe and is reported as the most extensively distributed pathogenic tick-borne virus (4). The worldwide distribution of HAZV has not been widely scrutinized, although antibodies against HAZV have been

Citation Kalkan-Yazıcı M, Karaaslan E, Çetin NS, Hasanoğlu S, Güney F, Zeybek Ü, Doymaz MZ. 2021. Cross-reactive anti-nucleocapsid protein immunity against Crimean-Congo hemorrhagic fever virus and Hazara virus in multiple species. J Virol 95:e02156-20. https://doi.org/10.1128/JVI.02156-20.

Editor Kanta Subbarao, The Peter Doherty Institute for Infection and Immunity

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Received 6 November 2020
Accepted 3 January 2021
Accepted manuscript posted online 13 January 2021
Published 10 March 2021

identified in wild rodent sera (5). Currently, there is neither prophylactic treatment nor an approved vaccine against CCHFV. On the other hand, nonpathogenic HAZV is not a biohazard for humans and can be studied under biosafety level 2 (BSL2) conditions. Possible serological and phylogenetic similarities between these two viruses offer a unique opportunity for studies on CCHFV, which requires a BSL4 facility.

The genomes of HAZV and CCHFV consist of three segments, namely, small (S), medium (M), and large (L) segments. The S segment encodes the nucleocapsid protein (NP), while the M segment encodes the Gn and Gc proteins within the glycoprotein precursor, and the L segment encodes viral RNA-dependent RNA polymerase (RdRp) (6).

The NP encoded by the S segment oligomerizes and encapsidates the three RNA segments in the genome, forming a ribonucleoprotein (RNP) complex. The RNP structure formed by the NP encapsulation of viral RNA (vRNA) plays an essential role in the entire life cycle of the virus, including replication, transcription, and virus assembly, and is moderated in orthonairoviruses through a close interaction between the RNP and viral glycoproteins (7). The participation of CCHFV NP in the replication of the virus has been demonstrated through the interaction of NP with the N-terminal regions of the L protein and colocalization with the labeled L protein in the transfected cells (8). In addition to the function of NP in replication and transcription machinery in orthonairoviruses, the NP's interaction with host immune element MxA protein is believed to contribute to immune evasion by the virus (9). NPs are the most conserved proteins among the other viral proteins in *Nairoviridae*, proving to be highly immunogenic, and are significant targets for both B and T lymphocytes in mammals (10). Detailed investigations are lacking on the immunological characterizations of HAZV NP, and there is a need for such scrutiny on these aspects of HAZV NP. Therefore, determining the antigenic similarities between CCHFV NP and HAZV NP may provide answers to the questions of whether the two proteins share antigenic similarities and whether they could substitute for each other in vaccine and immunology studies.

The amino acid identity of the sequences of the S segments between the CCHFV and the HAZV JC280 is 60% (11). The nucleotide differences among CCHFV isolates have been reported to be around 20% to 30%, depending on the segment (3). Therefore, the genetic similarities in the segments between HAZV and CCHFV are of notable interest. An RNA hybridization study performed decades earlier demonstrated similarities between S segments of these two viral strains, and later nucleotide sequence data supported these early reports (11). Other studies conducted in the 1980s showed the presence of cross-reaction between the two viruses by hemagglutination inhibition and neutralization tests (12). An unpublished observation reported by Foulke et al. indicated that HAZV provides cross-protection in mice after the CCHFV challenge (13). However, these early observations were not corroborated by others and were not followed up by supporting studies, after CCHFV became a worldwide epidemic. Currently, CCHFV is considered to have the potential to infect billions of people worldwide, and the similarities between HAZV and CCHFV deserve to be addressed in a methodical approach.

In this report, HAZV JC280 NP was used as a model to investigate the antigenic similarities with CCHFV. The aim of the study was to probe cross-reactivity in humoral immunity raised against HAZV and CCHFV in multiple species, namely, human, mouse, and rabbit. This study represents a detailed attempt to investigate the antigenic relationship between the two viruses and to assess the feasibility of utilizing HAZV as a model for CCHFV infections, for which preventive approaches are direly needed.

RESULTS

Generation of HAZV NP gene-encoding plasmid and expression of HAZV rNP. To generate highly purified HAZV recombinant NP (rNP) as an antigen in

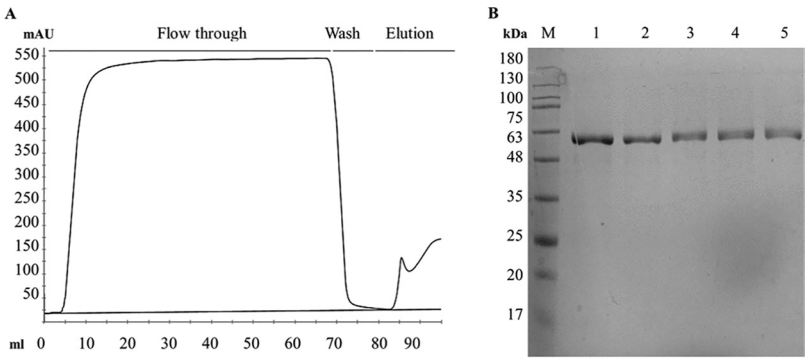

FIG 1 Purification of HAZV rNP by Ni-nitrilotriacetic acid (NTA) affinity chromatography. (A) Chromatogram of 6×His-tagged HAZV rNP monitored by UV 280-nm absorbance. (B) Analysis of elution fractions with Coomassie-stained 12% Tris-glycine SDS-PAGE gel. M, marker; 1 to 5, elution fractions.

immunizations, enzyme immunoassays (EIAs), and Western blots, a C-terminal His-tagged HAZV rNP was passed over nickel affinity chromatography (Fig. 1A). The purity of fractions was analyzed by SDS-PAGE (Fig. 1B), and ultrapure fractions were collected and dialyzed against phosphate-buffered saline (PBS) to use as an antigen. The identification of HAZV rNP was tested by Western blotting with an anti-His antibody (Fig. 2A).

Cross-reaction of HAZV rNP with CCHFV convalescent-phase human sera. In the study, 20 CCHFV-positive and 20 CCHFV-negative serum samples, confirmed with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Vector-Best, Novosibirsk, Russia), were tested to determine the cross-reactivity with HAZV rNP. In-house-developed HAZV rNP- and CCHFV rNP-based EIAs were optimized through a series of checkerboard EIAs testing antigen concentrations and secondary antibody (Ab) dilutions. The final conditions, as described in Materials and Methods, were then used to examine CCHFV-positive and -negative samples. Of the samples tested, the HAZV rNP EIA detected a total of 16 of 20 CCHFV-specific antibody-positive serum samples as positive and 18 of 20 CCHFV-specific antibody-negative samples as negative. The standard deviation (SD) calculation was based on the mean absorbance of 18 true-negative samples, and the SD values were determined to be 0.0181 and 0.028 for the HAZV rNP-based and CCHFV rNP-based EIAs, respectively. The mean absorbance values of the true-negative samples were 0.055 and 0.068 for HAZV rNP-based and CCHFV rNP-based EIAs, respectively. Finally, the cutoff values were calculated with the formula 2 × SD plus the mean absorbance of true negatives and were determined as 0.091 and 0.125 for HAZV rNP-based and CCHFV rNP-based EIAs, respectively. Data from the HAZV rNP-based EIA demonstrated that readings from serum samples in 16 of 20 patients were higher than the cutoff value, indicating positivity, while in the CCHFV rNP-based EIAs, samples from 18 of 20 patients produced reaction results higher than the cutoff value (Fig. 3A and B). The sensitivity and specificity of the HAZV rNP-based EIA in detecting CCHFV antibody-positive and -negative human sera were 80% and 90%, respectively. On the other hand, the sensitivity and specificity of the CCHFV rNP-based EIA in detecting CCHFV antibody-positive and -negative human sera were both 90%. These results demonstrated that there is a convincing antigenic similarity and cross-reactivity between NP's of HAZV and CCHFV as tested in convalescent-phase sera from CCHFV-infected subjects. Each data point was calculated by averaging the optical density values obtained from 3 independent experiments performed in duplicates of the absorbance readings at 450 nm. Grouped EIA data were compared by a Mann-Whitney U test and found to be significant ($P < 0.0001$).

Antigenic similarity of HAZV NP and CCHFV NP in multiple species. The reactions of HAZV rNP with antibodies found in CCHFV-immunized rabbit and mice and CCHFV-NP-immunized mouse sera were tested by Western blotting and in-house EIAs. The anti-CCHFV mouse sera and inactive CCHFV Kelkit06 viral strain were obtained

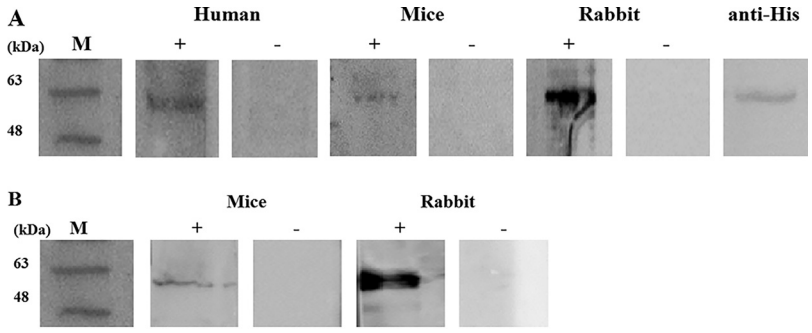

FIG 2 Detection of cross-reaction by Western blotting. (A) Purified HAZV rNP was run on 12% SDS-PAGE gels and transferred to nitrocellulose membranes. CCHFV-immunized/infected mouse and human sera, CCHFV-NP-immunized mouse sera, and anti-His tag antibodies were used. +, CCHFV infected or immunized; −, healthy or naive. (B) HAZV-infected and uninfected cell lysates were separated at equal amounts on 12% polyacrylamide gels by SDS-PAGE and transferred to nitrocellulose membranes. CCHFV-immunized mouse and rabbit sera were used as primary antibodies. M, marker; +, HAZV infected; −, HAZV uninfected cell lysate.

from Aykut Özdarendeli of Erciyes University. The anti-CCHFV rabbit sera were generated by immunization of white New Zealand adult rabbits with inactivated CCHFV, while anti-CCHFV-NP mouse sera were produced by immunization of BALB/c mice with CCHFV-NP (E. Karaaslan, N. S. Çetin, M. Kalkan-Yazici, S. Hasanoğlu, F. Güney, F. Karakeçili, A. Özdarendel, A. Kalkan, A. O. Kiliç, M. Z. Doymaz, submitted for publication). To investigate the antigenic relationship between CCHFV and HAZV, cross-reactivity of these viruses was first tested in Western blot experiments, where anti-CCHFV mouse and rabbit and anti-CCHFV-NP mouse sera were used as primary antibodies (Fig. 2A). In these assays,

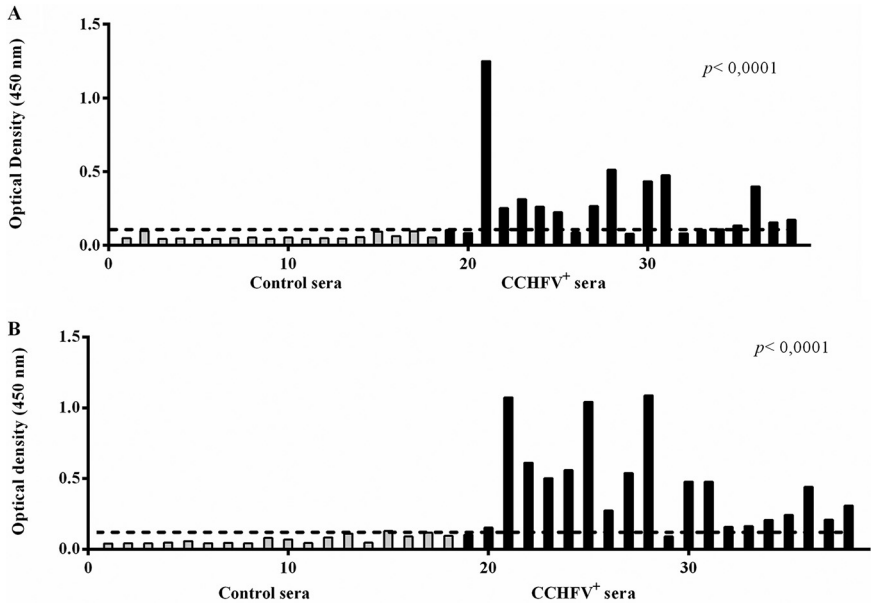

FIG 3 Detection of cross-reaction by EIA by using CCHF convalescent-phase human sera. EIA plates were coated with 0.1 μg/well ultrapure HAZV rNP and, as a positive control, with 0.1 μg/well ultrapure CCHFV rNP and analyzed using CCHF convalescent-phase and healthy human sera. Twenty CCHFV-positive and 20 CCHFV-negative serum samples were used for both EIAs. Standard deviation (SD) was calculated based on the OD values of 18 true-negative samples, and the cutoff value was calculated with the formula 2 × SD plus mean value. (A) Results from 16 of 20 patients remained higher than the cutoff value for HAZV rNP EIA. The sensitivity and specificity of HAZV rNP EIA is 80% and 90%, respectively. (B) Results from 18 of 20 patients remained higher than the cutoff value for CCHFV rNP EIA. The sensitivity and specificity of CCHFV rNP EIA is 90%. Each data point was calculated by averaging the optical density values obtained from three independent experiments performed in duplicates at an absorbance value of 450 nm. EIA data are compared by Mann-Whitney U test ($P < 0.0001$).

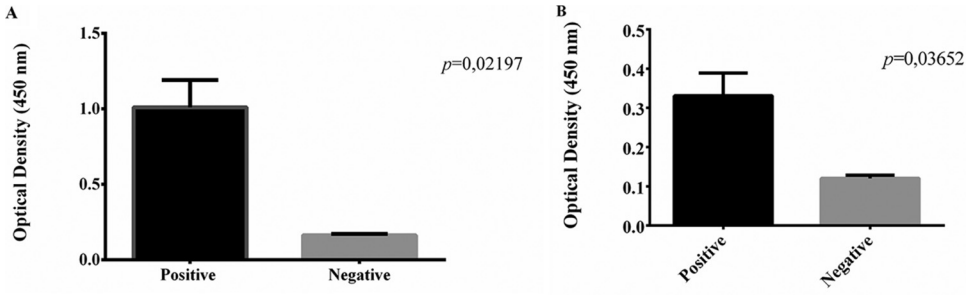

FIG 4 Detection of cross-reaction in cross-species by EIA. (A) Plates were coated with 10 μg/well ultrapure HAZV rNP and analyzed against 200-fold-diluted CCHFV-immunized rabbit sera. (B) Plates were coated with 1 μg/well and analyzed against 100-fold-diluted CCHFV-immunized mouse sera. Unprimed rabbit and mouse sera were used at the same dilutions as for the positive controls. Each data point was calculated by averaging the optical density values obtained from three independent experiments performed in duplicates at an absorbance value of 450 nm. Standard deviation (SD) was calculated based on the OD values of negative samples, and the cutoff value was calculated with formula 2 $\times$ SD plus mean value. OD values of positive samples remained higher than the cutoff value. The cutoff values were 0.176 and 0.131 for inactive-CCHFV-immunized rabbit and mouse EIA, respectively. EIA data were compared with two-tailed unpaired *t* test and found to be significant.

HAZV-infected cell lysate and uninfected BHK-21 cell lysates were used at equal protein concentrations as antigen in the Western blot experiments, and a 55-kDa protein band was detected on the gels in all positive serum samples, verifying antigenic cross-reactivities against NPs of both viruses (Fig. 2B). Similarly, in EIA, we used HAZV rNP as the antigen for testing anti-CCHFV rabbit and mouse sera as primary antibodies (Fig. 4A and B). Antibody titrations in EIA were performed with CCHFV-NP-immunized mouse sera, in that HAZV rNP (test antigen) and CCHFV rNP (positive control) were used as solid-phase antigens. Twofold serially diluted anti-CCHFV NP mouse sera were tested with the initial dilution 1/1,000, and CCHFV rNP and HAZV rNP were able to capture antibodies in sera even at a dilution of 1/32,000 (Fig. 5A and B). Data were calculated by averaging the optical density values obtained from three independent experiments performed in duplicates at an absorbance value of 450 nm. EIA data were compared with a two-tailed unpaired *t* test and found to be significant.

DISCUSSION

The data presented in the present study demonstrated undeniable antigenic similarities between HAZV and CCHFV in the humoral immune responses in multiple species. The strong cross-reaction between the NPs of HAZV and CCHFV were

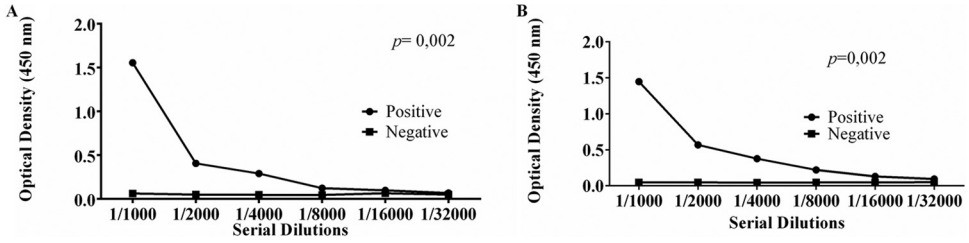

FIG 5 Titration curve EIA using CCHFV-rNP-immunized mouse sera. (A) Plates were coated with 1 μg/well ultrapure HAZV rNP, with 1 μg/well CCHFV rNP as a positive control. (B) EIA was performed using CCHFV-rNP-immunized mouse sera. Positive and negative serum samples were serially diluted 2-fold with the initial dilution of 1/1,000, and HAZV rNP and CCHFV rNP were able to capture antibodies in sera even at a dilution of 1/32,000. Each data point was calculated by averaging the optical density values obtained from three independent experiments performed in duplicates at an absorbance value of 450 nm. Standard deviation (SD) was calculated based on the OD values of negative samples, and the cutoff value was calculated with formula 2 $\times$ SD plus mean value. OD values of positive samples in each dilution remained higher than the cutoff value. The cutoff values were 0.069 and 0.053 for HAZV rNP and CCHFV rNP EIA, respectively. EIA data were compared with two-tailed unpaired *t* test and found to be significant.

documented in rabbit and mouse polyclonal antiserum raised against whole virus of CCHFV and NP of CCHFV as well as in human sera from patients that had recovered from CCHFV infection. Confirmation of cross-reactivity between NP's of these agents was proven by using HAZV rNP as an antigen in EIA and Western blotting with the above-listed antibodies. We also demonstrated the antigenic authenticity of the NPs by using inactive CCHFV, and CCHFV-NP-immunized mouse sera by conducting Western blotting utilizing HAZV-infected cell lysate as antigen. Hence, this study is the first to report similarity in anti-NP humoral immune responses between these two viruses.

The high mortality rate of the disease and the lack of an accepted treatment and vaccine make CCHFV a severe threat to public health. The World Health Organization declared CCHFV a high-priority pathogen (14) which requires handling in a BSL4 laboratory. However, the genetically related HAZV is not considered a human pathogen and could be studied under BSL2 conditions. HAZV was isolated from *Ixodes redikorzevi*-type ticks in the Hazara region of Pakistan for the first time in 1964 and classified in the same serogroup as CCHFV (1). HAZV might represent a potential surrogate virus for CCHFV. Nevertheless, the biology and immunology of HAZV have not been scrutinized in depth (15, 16).

The antigenic similarities between HAZV and several other nairoviruses, including CCHFV, were addressed several decades ago; curiously, no further confirmatory investigations ensued. A study by Casals and Tignor (12), which was conducted in 1980, examined the structural features of nairoviruses. This study indicates the cross-reaction between CCHFV and HAZV through neutralization and hemagglutination inhibition (12). In another study in 1980, Foulke et al. stated an unpublished observation that HAZV provided cross-protection for CCHFV in mice (13). Within the next decade of these reports, the sequence similarity between the S segments of the two viruses and the presence of a weak antigenic cross-reaction was demonstrated in a study by Marriott and Nuttall in 1992 (11). A recent report indicates that CCHFV and HAZV show similar pathology in immunocompromised mice, and both viruses show a lethal effect in embryonated chicken eggs (17). Apart from these incomplete scrutinies, no in-depth analyses have addressed the potential utility of HAZV against CCHFV. The reported studies are quite limited and are not sufficient to document conclusively that HAZV and CCHFV share antigenic similarities at the individual protein levels and, furthermore, that HAZV could serve as a comprehensive model for CCHFV. This present study using the NP of the HAZV JC280 strain was conducted to address this precise point.

In many RNA viruses, viral NPs can stimulate the humoral and cellular immune responses (18). The NPs represent the most predominant structural protein, with many functions in RNA viruses, and forms the RNP structure in mature virions (19). The crystal structure of HAZV and CCHFV NPs are structurally and chemically similar, and the nucleoproteins of both viruses superpose very closely, carrying a globular and two-helical-arm domain joined by a flexible hinge region (20). In both viruses, NPs interact with the heat shock protein 70 family, which plays an active role in virus growth (21). Proteomic studies with HAZV NP have shown that NP is involved in dynamic cell growth and fundamental cellular processes, such as the cell's defense mechanism, cell metabolism, and morphology (15). Also, in *Bunyavirus* infections, the NP stimulates the T and B cell responses because it has recognized a dominant antigen. Against many viruses, such as Ebola, Hanta, measles, and Rift Valley viruses, the NP has been used as an antigenic target in vaccine formulations and has been demonstrated to be protective (22–24). In this report, we developed a CCHFV-induced IgG-specific EIA using HAZV rNP with 80% sensitivity and 90% specificity and a CCHFV rNP-based EIA with 90% sensitivity and 90% specificity. This assay proved a strong cross-reaction between the two viruses in the CCHF-recovered patient population. The detection of antibodies against CCHFV-NP makes the NP a valuable antigen for clinical diagnosis (25, 26). Moreover, the antigenic similarity between NPs of these viruses, as detected in the

humoral immune response in multiple species, demonstrates that NP has similar antigenic epitopes and carries an appropriate antigenic structure for B cells. Antibodies against internal proteins such as NP may not have virus-neutralizing activity by themselves but may stimulate a beneficial immune response against infection, such as complement-mediated cell lysis, increased dendritic cell function, and reduced viral replication (27). Together with these results, it could be argued that there is a substantial NP-specific antibody response during the natural course of infection, and $CD4^+$ T cells recognize both NPs in multiple species. These data also imply that HAZV could serve as a surrogate for studying the humoral and cellular immune response generated by CCHFV. Furthermore, the data presented here also support the notion that HAZV could be used as a model agent for CCHFV, where experiments could be carried out safely under BSL2 conditions.

One of the limitations of our study is related to the epitopes of the antigenic proteins. It would be ideal to map the precise epitopes of the respective viral proteins that have been studied. These data would provide a structural basis for the similarities in antigenicity. The other point that should be addressed is how much the denaturation and refolding of the recombinant proteins influence the antigenic similarities between HAZV and CCHFV. These points and the actual proof of antigenic similarities in *in vivo* models await future investigations.

In conclusion, the present study examined the similarity between the NPs of the two viruses in terms of the humoral immune response. The other arm of adaptive immune response, namely, T cell response triggered by two NPs, could be addressed as a follow-up, and the potential of HAZV NP in vaccine efforts against CCHFV could be scrutinized in detail.

MATERIALS AND METHODS

Cell culture and virus. Baby hamster kidney-21 (BHK-21) cells were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. BHK-21 cells were infected with HAZV strain JC280 at a multiplicity of infection (MOI) of 0.01. After 3 days, virus-containing supernatants were harvested by centrifugation. Virus-containing cell supernatant was concentrated with 20% sucrose cushion in TNE buffer (20 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA) using an SW32Ti rotor at a speed of 150,000 $\times$ *g* for 4 h. The pellet was resuspended in 1 ml TNE buffer, aliquoted, and stored at −80°C for use as virus stock.

RNA isolation, cDNA synthesis, and reverse transcription-PCR. To obtain HAZV NP-encoding cDNA, total viral RNA was isolated from purified HAZV using GeneJET RNA purification kits, according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed using a ProtoScript II first-strand cDNA synthesis kit (New England Biolabs, MA, USA), according to the manufacturer's instructions. HAZV NP-encoding DNA sequence (1,455 bp) was amplified using 2 μl of cDNA as the template, and the PCR fragment was produced using gene-specific primers with the hot-start Phusion polymerase (New England Biolabs, NEB) with HF buffer. The sequences of the primers used in this study are as follows: forward primer, 5′-GGAATTCCATATGGAGAACAAGATTGTTGC-3′; reverse primer. 5′-CCGCTCGAGGATGATGTTGATGTTGGTG-3′.

Plasmid construction. For expression, HAZV NP was subcloned into pET-30b(+) (Addgene, Cambridge, MA, USA) using NdeI and XhoI restriction enzymes (New England Biolabs, MA, USA) and transformed into One Shot BL21(DE3) chemically competent *Escherichia coli* cells (Thermo Fisher Scientific, Waltham, MA, USA). After transformation, positive transformants were selected with kanamycin, confirmed by NdeI and XhoI restriction enzyme digestion, and further analyzed by sequencing (data not shown).

IPTG (isopropyl-β-D-thiogalactopyranoside)-induced expression of HAZV rNP in *E. coli* was performed. The most recombinant protein was expressed at 3 h postinduction, as identified by SDS-PAGE (data not shown). Bacterial cells were collected, and recombinant protein was detected in insoluble fractions. Therefore, the denaturing conditions were applied for purification.

For CCHFV NP production, the nucleotide sequence of CCHFV Kelkit06 NP (1,449 bp) retrieved from GenBank (accession no. GQ337053) was optimized for bacterial expression, synthesized, and cloned into pUC19 (GenScript USA Inc., Nanjing, China). CCHFV rNP was expressed and purified under the same conditions as for HAZV rNP as described in Materials and Methods and used as a positive control for EIA (Karaaslan et al., submitted).

Purification of His-tagged protein under denaturing conditions. An overnight culture with LB containing 50 μg/ml kanamycin was prepared for one selected positive transformant. When the optical density at 600 nm (OD_{600}) reached 1 to 2, the overnight culture was diluted 1:50 in LB and incubated at 37°C with 200 rpm to an OD_{600} 0.4 to 0.6. The culture was induced with IPTG (Biomatik, Cambridge, ON, Canada) at a final concentration of 1 mM when the OD_{600} of the bacterial culture reached 0.5, and the

culture was grown for another 3 h at 37°C on a rotary incubator shaker at 200 rpm. Cells were harvested by centrifugation at 5,000 × *g* for 5 min at 4°C and washed with phosphate-buffered saline (PBS). Thereafter, cells were resuspended in lysis buffer (10 mM Tris-HCl, 8 M urea, 1% Tween 20, 300 mM NaCl, pH 8.5), freeze-thawed 3 times, sonicated 3 times on ice for 10-s bursts (Sonopuls HD 2070; BANDELIN Electronic GmbH & Co. KG, Berlin, Germany), and incubated at room temperature with agitation for 2 h. Lysed cells were clarified for purification at 30,000 × *g* for 30 min. Clear lysates were used to purify His-tagged HAZV rNP. Purification of rNP was performed on an ÄKTA pure chromatography system (GE Healthcare Life Sciences, Glattbrugg, Switzerland) using HisTrap Excel columns (GE Healthcare Life Sciences) under denaturing conditions. The fractions were eluted with lysis buffer containing 250 mM imidazole, and the purity of fractions was analyzed by 12% SDS-PAGE. To refold pure denatured proteins, dialysis was conducted at room temperature against PBS using SnakeSkin dialysis tubing (Thermo Fisher Scientific, Waltham, USA) for use in EIA and Western blotting. Protein amounts were determined both by a Bradford protein assay kit (Thermo Fisher Scientific), according to manufacturer's instructions, and by absorbance values measured at 280 nm on a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific).

SDS-PAGE and Western blotting. The fractions for SDS-PAGE and Western blotting were prepared by the addition of Laemmli buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl) and incubated at 95°C for 10 min. Equal amounts of samples were loaded onto a 12% SDS-PAGE gel and separated by electrophoresis. The gels for SDS-PAGE analysis were subsequently stained with 0.1% Coomassie brilliant blue R-250 (Merck, KGaA, Darmstadt, Germany) prepared in methanol.

Following viral infection, the expressed and purified HAZV rNP and HAZV NP in BHK-21 were verified by Western blotting. Equal amounts of pure HAZV rNP were separated on a 12% PAGE gel. HAZV-infected BHK-21 cell lysate and uninfected BHK-21 cell lysate were also loaded onto the gel in the same amounts as a control. Then, the gels were electroblotted onto 0.45-μm nitrocellulose (Amersham Protran) at 25 V for 7 min using a Trans-blot SD semidry blotter (Bio-Rad). The membrane was blocked in blocking buffer (5% nonfat dairy milk and 1× TBST [10 mM Tris-HCl {pH 7.4}, 0.9% NaCl, 0.02% Tween 20]) for 2 h and probed overnight with primary antibodies at 4°C. Primary antibodies were prepared in blocking buffer at a dilution of 1:1,000 for CCHFV-immunized mouse sera, at a dilution of 1:3,000 for CCHFV-NP-immunized mouse sera, at a dilution of 1:10 for CCHFV-immunized rabbit sera, and at a dilution of 1:4000 for CCHFV-infected human sera. As negative controls, nonimmunized rabbit and mouse sera and healthy human sera were used at the same dilutions. To visualize the specific antigen-antibody reaction on the membrane, horseradish peroxidase-conjugated goat anti-mouse IgG (sc-2005; Santa Cruz Biotechnology, Inc. Dallas, TX, USA), goat anti-rabbit IgG (sc-2030; Santa Cruz Biotechnology, Inc.), goat anti-human IgG (5172-2504; Bio-Rad Laboratories, Inc., Hercules, CA, USA), and anti-6×His tag antibody (HRP; Abcam, UK) were used as secondary antibodies at dilutions suggested by the manufacturers. Finally, the membrane was washed five times and visualized via chemiluminescence detection (WesternBright Sirius chemiluminescence detection kit; Advansta, CA, USA), according to the manufacturer's instructions.

In-house IgG EIA. Flat-bottomed polystyrene 96-well microtiter plates (Immulon1B; Dutscher Scientific) were coated with antigens in coating buffer (35 mM $NaHCO_3$ [1,465 mg], 15 mM Na_2CO_3 [765 mg] in 500 ml distilled water, pH 9.6) overnight at 4°C. When testing human sera, 0.1 μg/well of HAZV rNP and CCHFV rNP were used as antigens. When testing immunized mouse serum in EIA, the concentrations of antigens were 1 μg/well. In the rabbit serum tests, the concentrations were 10 μg/well. Antibody titration EIA was performed with serially diluted CCHFV-NP-immunized mouse sera. The plates were blocked with 200 μl 5% nonfat dairy milk in PBS with 0.2% Tween 20 (PBST; pH 7.2) for 2 h at room temperature (RT). Then, the wells were aspirated, and either 100 μl of 100-fold dilution of the polyclonal anti-CCHFV mouse sera, 100 μl of 200-fold diluted anti-CCHFV rabbit sera, or 100 μl of 100-fold diluted CCHFV-infected human sera in 5% nonfat dairy milk in PBST was added and incubated for 1 h at 37°C. As negative controls, naive rabbit and mouse sera and healthy human sera were used at the same dilutions. The original diagnoses of the infections in all positive subjects were made by the Public Health Laboratories of the Turkish Ministry of Health with a reverse transcription-PCR test directed to CCHFV and were further confirmed by a commercial serological CCHF-IgG EIA (Vector-Best, Novosibirsk, Russia). In the experiments, 20 CCHFV-specific antibody-positive and 20 CCHFV-specific antibody-negative human serum samples were used. All experiments were performed in triplicates. The plates were washed 3× with PBST and incubated for 1 h at 37°C with 100 μl of a 1/10,000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (5172-2504; Bio-Rad Laboratories, Inc.), 1/1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (sc-2005; Santa Cruz Biotechnology Inc.), or 1/5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (sc-2030; Santa Cruz Biotechnology, Inc.). The plates were washed 3× with PBST, and 100 μl 3,3′,5,5′-tetramethylbenzidine (TMB; Abcam, Cambridge, UK) substrate was added per well. The plates were incubated in the dark at 37°C for 10 min, and 100 μl of 2 N H_2SO_4 was added to each well to stop the reaction. The absorbance was measured in an iMark microplate reader (Bio-Rad Laboratories, Inc.) at 450 nm. All washing steps were performed using a Wellwash Versa microplate washer (Thermo Fisher Scientific, Waltham, USA). The mean values ± standard deviations (SDs) are presented from 3 independent experiments performed in duplicates.

Statistical analyses. The statistical analyses were performed with Prism 6 for OS X version 6.0.1 (GraphPad Software, San Diego, CA). Significance between grouped data was determined by Wilcoxon or Mann-Whitney U tests using Prism 6.0 (GraphPad) or a two-tailed Student's *t* test for unpaired samples. Significance for all comparisons was accepted at a *P* value of <0.05 value.

Ethical clearance. The study protocols of both human and animal experiments have received ethical clearance from the Ethics Committee for Non-Invasive Studies Involving Human Subjects and the Ethics Committee for Animals Studies of Bezmialem Vakif University in Istanbul, Turkey (approval numbers 08/137 and 4.2019/12).

ACKNOWLEDGMENTS

We declare that no conflict of interest exists.

The funding for the study was provided by the Scientific Research Unit of Bezmialem Vakif University (no. 6.2017/27 and 4.2019/12).

REFERENCES

1. Begum F, Wisseman CL, Jr, Casals J. 1970. Tick-borne viruses of West Pakistan. II. Hazara virus, a new agent isolated from *Ixodes redikorzevi* ticks from the Kaghan Valley, W. Pakistan. Am J Epidemiol 92:192–194. https://doi.org/10.1093/oxfordjournals.aje.a121197.
2. Honig JE, Osborne JC, Nichol STJV. 2004. The high genetic variation of viruses of the genus *Nairovirus* reflects the diversity of their predominant tick hosts. Virology 318:10–16. https://doi.org/10.1016/j.virol.2003.09.021.
3. Bente DA, Forrester NL, Watts DM, McAuley AJ, Whitehouse CA, Bray M. 2013. Crimean-Congo hemorrhagic fever: history, epidemiology, pathogenesis, clinical syndrome and genetic diversity. Antiviral Res 100:159–189. https://doi.org/10.1016/j.antiviral.2013.07.006.
4. Spengler JR, Bergeron E, Spiropoulou CF. 2019. Crimean-Congo hemorrhagic fever and expansion from endemic regions. Curr Opin Virol 34:70–78. https://doi.org/10.1016/j.coviro.2018.12.002.
5. Hoogstraal H. 1979. The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa. J Med Entomol 15:307–417. https://doi.org/10.1093/jmedent/15.4.307.
6. Morikawa S, Saijo M, Kurane I. 2007. Recent progress in molecular biology of Crimean-Congo hemorrhagic fever. Comp Immunol Microbiol Infect Dis 30:375–389. https://doi.org/10.1016/j.cimid.2007.07.001.
7. Carter SD, Surtees R, Walter CT, Ariza A, Bergeron E, Nichol ST, Hiscox JA, Edwards TA, Barr JN. 2012. Structure, function, and evolution of the Crimean-Congo hemorrhagic fever virus nucleocapsid protein. J Virol 86:10914–10923. https://doi.org/10.1128/JVI.01555-12.
8. Bergeron E, Albarino CG, Khristova ML, Nichol ST. 2010. Crimean-Congo hemorrhagic fever virus-encoded ovarian tumor protease activity is dispensable for virus RNA polymerase function. J Virol 84:216–226. https://doi.org/10.1128/JVI.01859-09.
9. Andersson I, Bladh L, Mousavi-Jazi M, Magnusson KE, Lundkvist A, Haller O, Mirazimi A. 2004. Human MxA protein inhibits the replication of Crimean-Congo hemorrhagic fever virus. J Virol 78:4323–4329. https://doi.org/10.1128/jvi.78.8.4323-4329.2004.
10. Zivcec M, Scholte FEM, Spiropoulou CF, Spengler JR, Bergeron E. 2016. Molecular Insights into Crimean-Congo hemorrhagic fever virus. Viruses 8:106. https://doi.org/10.3390/v8040106.
11. Marriott AC, Nuttall PA. 1992. Comparison of the S-RNA segments and nucleoprotein sequences of Crimean-Congo hemorrhagic fever, Hazara, and Dugbe viruses. Virology 189:795–799. https://doi.org/10.1016/0042-6822(92)90609-s.
12. Casals J, Tignor GH. 1980. The *Nairovirus* genus - serological relationships. Intervirology 14:144–147. https://doi.org/10.1159/000149175.
13. Foulke RS, Rosato RR, French GR. 1981. Structural polypeptides of Hazara virus. J Gen Virol 53:169–172. https://doi.org/10.1099/0022-1317-53-1-169.
14. World Health Organization. 2018. Roadmap for research and product development against Crimean-Congo haemorrhagic fever (CCHF). http://www.who.int/blueprint/priority-diseases/key-action/cchf-draft-r-and-d-roadmap.pdf.
15. Dowall SD, Findlay-Wilson S, Rayner E, Pearson G, Pickersgill J, Rule A, Merredew N, Smith H, Chamberlain J, Hewson R. 2012. Hazara virus infection is lethal for adult type I interferon receptor-knockout mice and may act as a surrogate for infection with the human-pathogenic Crimean-Congo hemorrhagic fever virus. J Gen Virol 93:560–564. https://doi.org/10.1099/vir.0.038455-0.
16. Smirnova SE, Shestopalova NM, Reingold VN, Zubri GL, Chumakov MP. 1977. Experimental Hazara virus infection in mice. Acta Virol 21:128–132.
17. Matsumoto Y, Ohta K, Nishio M. 2018. Lethal infection of embryonated chicken eggs by Hazara virus, a model for Crimean-Congo hemorrhagic fever virus. Arch Virol 163:219–222. https://doi.org/10.1007/s00705-017-3580-1.
18. Ding BB, Qin YL, Chen MZ. 2016. Nucleocapsid proteins: roles beyond viral RNA packaging. Wiley Interdiscip Rev RNA 7:213–226. https://doi.org/10.1002/wrna.1326.
19. Zhou H, Sun Y, Guo Y, Lou Z. 2013. Structural perspective on the formation of ribonucleoprotein complex in negative-sense single-stranded RNA viruses. Trends Microbiol 21:475–484. https://doi.org/10.1016/j.tim.2013.07.006.
20. Surtees R, Ariza A, Punch EK, Trinh CH, Dowall SD, Hewson R, Hiscox JA, Barr JN, Edwards TA. 2015. The crystal structure of the Hazara virus nucleocapsid protein. BMC Struct Biol 15:24. https://doi.org/10.1186/s12900-015-0051-3.
21. Surtees R, Dowall SD, Shaw A, Armstrong S, Hewson R, Carroll MW, Mankouri J, Edwards TA, Hiscox JA, Barr JN. 2016. Heat shock protein 70 family members interact with Crimean-Congo hemorrhagic fever virus and Hazara virus nucleocapsid proteins and perform a functional role in the *Nairovirus* replication cycle. J Virol 90:9305–9316. https://doi.org/10.1128/JVI.00661-16.
22. Dowall SD, Graham VA, Rayner E, Hunter L, Watson R, Taylor I, Rule A, Carroll MW, Hewson R. 2016. Protective effects of a modified vaccinia Ankara-based vaccine candidate against Crimean-Congo haemorrhagic fever virus require both cellular and humoral responses. PLoS One 11:e0156637. https://doi.org/10.1371/journal.pone.0156637.
23. Boshra H, Lorenzo G, Rodriguez F, Brun A. 2011. A DNA vaccine encoding ubiquitinated Rift Valley fever virus nucleoprotein provides consistent immunity and protects IFNAR$^{-/-}$ mice upon lethal virus challenge. Vaccine 29:4469–4475. https://doi.org/10.1016/j.vaccine.2011.04.043.
24. Zivcec M, Safronetz D, Scott DP, Robertson S, Feldmann H. 2018. Nucleocapsid protein-based vaccine provides protection in mice against lethal Crimean-Congo hemorrhagic fever virus challenge. PLoS Negl Trop Dis 12:e0006628. https://doi.org/10.1371/journal.pntd.0006628.
25. Marriott AC, Polyzoni T, Antoniadis A, Nuttall PA. 1994. Detection of human-antibodies to Crimean-Congo hemorrhagic fever virus using expressed viral nucleocapsid protein. J Gen Virol 75:2157–2161. https://doi.org/10.1099/0022-1317-75-9-2157.
26. Emmerich P, Mika A, von Possel R, Rackow A, Liu Y, Schmitz H, Gunther S, Sherifi K, Halili B, Jakupi X, Berisha L, Ahmeti S, Deschermeier C. 2018. Sensitive and specific detection of Crimean-Congo hemorrhagic fever virus (CCHFV)-specific IgM and IgG antibodies in human sera using recombinant CCHFV nucleoprotein as antigen in mu-capture and IgG immune complex (IC) ELISA tests. PLoS Negl Trop Dis 12:e0006366. https://doi.org/10.1371/journal.pntd.0006366.
27. Yewdell JW, Frank E, Gerhard W. 1981. Expression of influenza A virus internal antigens on the surface of infected P815 cells. J Immunol 126:1814–1819.