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Shabnam Ansari

Translational Health Science and Technology Institute, NCR Biotech Science Cluster

Jyoti Gupta

Translational Health Science and Technology Institute, NCR Biotech Science Cluster

Geetika Anand

Manav Rachna International Institute of Research and Studies

Mohd Faiz Saifi

All India Institute of Medical Sciences

Shivani Pandey

All India Institute of Medical Sciences

Priyanka Sharma

All India Institute of Medical Sciences

Tejeswara Rao Asuru

RCB

Umang Berry

Translational Health Science and Technology Institute, NCR Biotech Science Cluster

Kannan Chandrasekar

Translational Health Science and Technology Institute, NCR Biotech Science Cluster

Amit Yadav

Translational Health Science and Technology Institute, NCR Biotech Science Cluster

Prasenjit Guchhait

RCB

Tripti Shirvastava

Translational Health Science and Technology Institute, NCR Biotech Science Cluster

Krishna Priya Ganti

Manav Rachna International Institute of Research and Studies

Sunil Taneja

Postgraduate Institute of Medical Education and Research

Kathirvel Soundappan

Postgraduate Institute of Medical Education and Research

Radha Kanta Ratho

Postgraduate Institute of Medical Education and Research

. Shalimar

All India Institute of Medical Sciences

Sanjay Rai

All India Institute of Medical Sciences

Baibaswata Nayak

All India Institute of Medical Sciences

Milan Surjit

milan@thsti.res.in

Translational Health Science and Technology Institute, NCR Biotech Science Cluster

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Proteome wide Mapping reveals a Neutralizing Epitope at the N-terminus of Hepatitis E Virus Capsid Protein with Implications for Vaccine Design

Shabnam Ansari¹, Jyoti Gupta^{1#}, Geetika Anand², Mohd Faiz Saifi³, Shivani Pandey³, Priyanka Sharma³, Tejeswara Rao Asuru⁴, Umang Berry¹, Kannan Chandrasekar¹, Amit Yadav¹, Prasenjit Guchhait⁴, Tripti Shirvastava⁵, Krishna Priya Ganti², Sunil Taneja⁶, Kathirvel Soundappan⁷, Radha Kanta Ratho⁸, Shalimar³, Sanjay Rai⁹, Baibaswata Nayak³, Milan Surjit^{1*}

¹Virology Laboratory, Centre for Virus Research, Therapeutics and Vaccines, Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad, Haryana, India.

²Manav Rachna International Institute of Research and Studies, Faridabad, India.

³Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi, India.

⁴Regional Centre for Biotechnology, National Capital Region Biotech Science Cluster, Faridabad, Haryana, India.

⁵Centre for Virus Research, Therapeutics and Vaccines, Translational Health Science and Technology Institute, NCR Biotech Science Cluster, Faridabad, Haryana, India.

⁶Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh, India.

⁷Department of Community Medicine and School of Public Health, Postgraduate Institute of Medical Education and Research, Chandigarh, India.

⁸Department of Virology, Postgraduate Institute of Medical Education and Research, Chandigarh, India.

⁹Center For Community Medicine, All India Institute of Medical Sciences, New Delhi, India.

Current address: Department of Biotechnology, GLA University, Mathura, India.

*Correspondence: milan@thsti.res.in

Abstract

Hepatitis E virus (HEV) causes acute viral hepatitis (AVH), fulminant hepatitis and community-level outbreaks. Chronic HEV infection is observed in many solid organ transplantation patients. The disease worsens during pregnancy, with ~30% mortality rate. Earlier studies using different fragments of the purified viral capsid protein have identified the region encompassing major neutralizing epitopes in HEV capsid. However, there is no systematic study regarding repertoire of B-cell epitopes in HEV. This study aimed at unbiased identification of B-cell epitopes in the human-infecting genotypes of HEV, with the goal of designing a more potent HEV vaccine. Proteome-wide mapping of B-cell epitopes present in human-infecting HEV genotypes by cyclic peptide microarray using HEV patient sera revealed the repertoire of B-cell epitopes in HEV. Importantly, HEV patient sera show highest reactivity against an epitope located between 116-126 aa of g1-HEV ORF2 protein. Synthetic peptides corresponding to the above epitope react with HEV-positive human sera. Recombinant 112-608 ORF2 protein immunized mice sera display higher anti-ORF2 IgG titre, virus neutralization titre (NT50) and memory T-cell response compared to that of the 368-606 ORF2. Furthermore, immunodepletion of the 112-608 ORF2 antigen immunized mice sera with synthetic peptide corresponding to 116-126 aa of g1-HEV ORF2 considerably reduces its anti-ORF2 antibody level and NT50 titres. In summary, we report a comprehensive data of B-cell epitopes in HEV and characterize a new neutralizing epitope at the N-terminus of 112-608 ORF2 protein, which contributes to its superior virus neutralization efficacy. These findings pave the way for development of next generation, more potent HEV vaccine.

Introduction

Hepatitis E virus (HEV) is a feco-orally transmitted pathogen that causes self-limiting acute hepatitis in most cases. Cases of chronic HEV infection in immunocompromised individuals have been reported (1, 2). Further, HEV infection in pregnant women may result in up to ~30% mortality (3). There are eight genotypes of HEV; genotypes 1 (g1) and 2 (g2) infect only humans, genotypes 3 (g3) and 4 (g4) infect humans, pigs, wild boars and deer. HEV genotypes 5 (g5) and 6 (g6) infect wild boars while HEV genotypes 7 (g7) and 8 (g8) infect dromedaries and Bactrian camel, respectively (4). Genotypes 1 and 2 prevail in parts of Asia and Africa, and are transmitted via contaminated food and water, whereas transmission of genotypes 3 and 4 occurs in the Europe and Americas (North and South) through consumption of undercooked or raw meat and blood transfusion (4, 5).

HEV is a positive sense single-stranded RNA virus with a genome size of around 7200 nucleotides. The viral genome contains a 7-methylguanosine cap at the 5'-terminus, 25-26 nt long 5'-UTR, followed by three open reading frames (ORFs) and a short polyadenylated 3'-UTR (6, 7). ORF1 encodes the non-structural polyprotein, involved in viral replication. The polyprotein contains several functional domains such as Methyltransferase (MeT), Y, Protease (PCP), V, X, Helicase (Hel) and RNA-dependent RNA polymerase (RdRp) domains (8). ORF2 encodes the major capsid protein. It self-associates to form the viral capsid and interacts with viral RNA (9, 10). Heterologous expression of the ORF2 protein in human hepatoma cells induces endoplasmic reticulum stress and inhibits cellular NF- κ B signaling pathway (11). ORF3 encodes a phospho-protein that modulates cell signaling, virus survival and release of the progeny virus (12). A fourth ORF (ORF4) has been identified in g1-HEV, which is expressed through internal ribosome entry-site (IRES) like element mediated translation, under ER-stress conditions. ORF4 promotes the assembly of proteins involved in viral replication and enhances viral replication (13).

Several HEV vaccine candidates have been investigated. All HEV genotypes show one serotype (14). Both humoral and T cell-mediated immune responses are important for HEV clearance (15). Being the capsid protein, most studies evaluated the immunogenicity and virus neutralization potential of vaccine candidates developed from different regions of the ORF2 protein as the antigen. ORF2 protein is 660 amino acid long, divided into three functional domains, such as shell (S), middle (M) and protruding (P) domains, located between 129–319aa, 320–455aa and 456–606aa, respectively. P-domain contains major neutralizing conformational epitopes and M-domain contains linear neutralizing epitopes between 403–417aa, 423–437aa and 443–457aa (overlapping with the P-domain) (16-18). Peptide-based mapping experiments has shown the presence of T-cell epitopes between 73-156aa, 289-372aa, 361-444aa, and 505-588aa of the ORF2 protein (19). A recent immunoinformatics study predicted the presence of a T-cell epitope between 53-61aa of the ORF3 protein and 7 T-cell epitopes between 159-170aa, 204-212aa, 212-224aa, 217-229aa, 277-284aa, 318-

326aa and 325-333aa of the ORF2 protein. Similarly, a B-cell epitope was found in the 69-74aa region of the ORF3 protein and 4 B cell epitopes were found between 160-166aa, 323-333aa, 444-449aa, 469-489aa of the ORF2 protein (20).

Hecolin is the only commercially available vaccine against HEV, which utilizes an *E.coli* expressed VLP of HEV ORF2 (368-606aa) as the vaccine antigen. The VLP in Hecolin is reconstituted from 368-606 ORF2 (purified from the inclusion bodies of *E.coli*), having an average diameter of 25 nm (21, 22). Initial studies showed that Hecolin vaccine was highly immunogenic in mice, induced strong Th1 and Th2 responses and protected rhesus monkeys against g1- or g4-HEV challenge (21-23). In a clinical trial, Hecolin vaccine protected against Hepatitis E for 4.5 years (24). The vaccine is currently under clinical trial IV and licensed for commercial use in China and Pakistan (25). Recombinant Hepatitis E-VLP vaccine candidates based on 112-608aa and 439-617aa regions of the ORF2 protein have also been reported (26-29). Notably, 112-608aa ORF2 purified from baculovirus infected insect cells formed a 270 Å diameter N-linked glycosylated VLP with T=1 symmetry (30). This VLP protected rhesus monkey from HEV challenge after 6 or 12 months of vaccination (27). Further, it showed a protection efficacy of 95% in a phase II clinical trial (28).

Here, we performed a 3D printing-based cyclic peptide microarray for B-cell epitope mapping of human serum samples against Hepatitis E antigens of human-infecting HEV genotypes (g1, g2, g3, g4, g7). Twenty one significantly immunogenic epitopes were identified in g1-HEV. Similarly, 13, 14 and 9 epitopes were identified in g2, g3 and g4-HEV, respectively. HEV positive sera showed highest reactivity with a previously unknown epitope located between 116-126aa of g1-HEV ORF2. Multiple assays using serum sample from 150 HEV sero-positive individuals and immunized mice sera confirmed immunogenicity and virus neutralization potential of the above epitope. Functional significance of the newly identified epitope in enhancing the protective efficacy of 112-608 ORF2 vaccine antigen is discussed.

Results

Proteome wide B-cell epitope mapping in human-infecting HEV genotypes

With informed consent, blood samples from three clinically confirmed adult HEV positive patients and three healthy volunteers (HEV negative) were obtained (Table 1). Three HEV-positive sera and three Healthy HEV-negative sera were pooled, respectively, and analysed by 3D printing-based cyclic peptide microarray for B-cell epitope mapping of Hepatitis E antigens of human-infecting HEV genotypes (g1, g2, g3, g4, g7). The sequences of ORF1, ORF3 and ORF2 of HEV genotypes 1, 2, 3, 4 and 7 were translated into 13 amino acid peptides with a peptide-peptide overlap of 11 amino acids. Each peptide sequence was linked and elongated by neutral GSGSGSG linkers at the C- and N-terminus to avoid truncated

peptides. Synthesized peptides were cyclized via a thioether linkage between a C-terminal cysteine and an appropriately modified N-terminus. The resulting conformational peptide microarrays contained 5,426 different peptides printed in duplicate (10,852 peptide spots) and were framed by additional HA (YPYDVDPDYAG, 24 spots), polio (EVPALTAVETGAT, 16 spots) and c-Myc (EQKLISEEDL, 24 spots) control peptides (Table S1 and S2, see “Peptide map” tabs).

Staining condition was optimized to minimize background interactions. Some very weak background interactions of the control antibody with highly basic peptides like QAQRPAVASRRRP, QSQRPTAAPRRRP or AGQPSGRRRRGRRS was observed, presumably due to less specific ionic binding and few cross-reactions with peptides like HSALAVLEDTADY or HSALAILEDTVDY due to sequence homology with the HA control peptides. However, these background interactions did not interfere with the analysis of the main assays in the red channel. Staining with the control antibody revealed well-defined HA control spot pattern framing the peptide microarray, validating the overall peptide microarray integrity and assay quality (Figure S1). Human serum samples were tested at 1:500 and 1:1000 dilutions in incubation buffer, the latter being better. Microarrays were incubated with the human serum samples at 1:1000 dilution in incubation buffer, followed by staining with Goat anti-human IgG (Fc) DyLight680 and image acquisition at scanning intensities of 7/7 (red/green). Quantification of microarray spot intensities and peptide annotation were based on the 16-bit gray scale tiff files (Table S1 and S2, see “Raw Data” tabs). Raw data were analyzed in PepSlide Analyzer (Pepperprint GmbH) to calculate the average median foreground intensities and spot-to-spot deviations of spot duplicates, permitting a maximum spot-to-spot deviation of 40% (otherwise the corresponding intensity value was zeroed) (Table S1 and S2, see “Mapping Summary” tabs). Based on averaged median foreground intensities, an intensity map was generated and interactions in the peptide map were highlighted by an intensity color code with red for high and white for low spot intensities (Figure S2). Averaged spot intensities of the assays with the human serum samples against the antigen sequences from the N-terminus of ORF1 (NSP) to the C-terminus of ORF2 (cap) were plotted to visualize overall spot intensities and signal-to-noise ratios (Table S3_ see “Intensity Plot” tabs). The intensity plots were correlated with peptide and intensity maps as well as with visual inspection of the microarray scans (Table S3, Figure S2). Intensity values above 500 were considered as significant. Intensity plot of 5426 peptides against HEV positive and Healthy HEV negative sera is shown (Figure 1A).

Twenty one conformational epitopes were detected in g1-HEV, 8 located in ORF1, 2 located in ORF3 and 11 located in ORF2 (Figure 1B). 13, 14 and 9 conformational epitopes were detected in g2-, g3a and g4-HEV, respectively (Figure S3). 3 peptides corresponding to capsid region of g3b- and g3c-HEV were also detected (Figure S3). Out of the 21 g1-HEV epitopes, 4 epitopes have been reported earlier [Figure 1B, C16, C18, C20 (boxed sequences)] and 7

are also present in one or more of other genotypes (Figure 1B, C3, C5, C9, C10, C15, C17, C19, C21) (20). Importantly, the epitope showing highest fluorescence intensity in g1-, g3 and g4- HEV genotypes and second highest fluorescence intensity in g2-HEV [Figure 1B, C15 (APAHDTPPVPDVD), 114-126aa of g1-HEV ORF2] is conserved, the consensus sequence being A[H/P]DT[P/S/A]PVPDVD (Figure 1C). It is located in the S-domain of the ORF2 protein. Another g1-HEV epitope with significant fluorescence intensity [C16 (NTPYTGALGLLDF, 290-302aa of g1-HEV ORF2)] is also located in the S-domain. The epitope showing second highest fluorescence intensity in g1-HEV [C21 (VVLAPHSALALLED, 600-612aa of g1-HEV ORF2)] is located in the P-domain. Remaining 4 epitopes with significant fluorescence intensity are also located in the P-domain (Figure 1B, C17-C20).

Two epitopes (C9, C10) are found at the C-terminal region of g1-HEV ORF3 protein. Out of the 8 epitopes present in ORF1, 3 are located in the PCP region (C2, C3, C4), 2 each are located in the Helicase (C7, C8) and macro domain (C5, C6) region and 1 epitope is located in the Y-domain (C1) region. One epitope is located in the macro domain region [C6 (DAWERNHRPGDELL, 900-912aa of g1-HEV ORF1)] (Figure 1B).

Table 1. Demographic, serological and clinical profile of HEV infected patient, HEV- exposed CLD patient and Healthy control

Sr. No	Variable	HEV infected Patient (AIIMS, n=15)	HEV infected Patient (PGIMER, n=33)	HEV exposed CLD Patient (AIIMS, n=102)	Healthy Control (AIIMS, n=6)
1	Age (Year) Mean $\pm$ SD	34.2 $\pm$ 17.3	30.4 $\pm$ 11.7	41.5 $\pm$ 10.5	40 $\pm$ 8.07
2	Sex (n, %) Male Female	10 (66.6 %) 5 (33.4%)	9 (27.3%) 24 (72.7%)	82 (80.4%) 20 (19.6%)	1 (16.66%) 5 (83.34%)
3	Diagnosis (n,%) HEV infected AVH ALF ACLF HEV Exposed CLD-CHB CLD-CHC CLD-MASLD CLD-BCS CLD -AIH CLD-MetALD CLD-Other liver diseases	6 (40%) 4 (26.6%) 5 (33.4%) - - - - - - -	26 (78.8%) 5 (15.2%) 2 (6.1%) - - - - - - -	- - - 45 (44.1%) 18 (17.6%) 8 (7.8%) 6 (5.9%) 5 (4.9%) 10 (9.8%) 10 (9.8%)	
4	Clinical status, n (%) Pregnant Death No special status/Other	- 2 (13.3%) 13	12 (36.4%) 2 (6.1%) 19 (57.6%)	- - 102 (100%)	- - 6 (100%)
5	HEV IgM titer Positive, n, % Negative, n % Mean $\pm$ SD Median (IQR)	8 (53.3%) 7 (46.7%) 3.7 $\pm$ 9.5 0.94 (0.61-34.12)	33 (100%) -	- 102 (100%)	- 6 (100%)
6	HEV IgG titer Positive, n, % Negative, n % Mean $\pm$ SD Median (IQR)	15 (100%) - 31.8 $\pm$ 36.19 15.43 (5.5-104.6)	33 (100%) -	102 (100%) - 35.19 $\pm$ 100.17 10.65 (7.86–25.45)	- 6 (100%)
7	HEV RNA Positive, n, % Negative, n % Mean $\pm$ SD (Log ₁₀ IU/ml)	8 (53.3%) 7 (46.7%) 1.97 $\pm$ 0.61	13 (39.4%) 20 (60.6%) 3.51 $\pm$ 0.81	Not Done	Not Done

AVH-Acute viral hepatitis, ALF-Acute Liver failure, ACLF-Acute on chronic liver failure, CLD-Chronic liver disease, CHB-Chronic Hepatitis B, CHC-Chronic Hepatitis C, MASLD-metabolic dysfunction-associated steatotic liver disease, BCS- Budd-Chiari syndrome, AIH-Autoimmune hepatitis, MetALD-Metabolic dysfunction and alcohol-associated liver disease

In order to evaluate the functional significance of the microarray data, we focused on further characterization of the epitope C15 that showed the highest fluorescence intensity in g1-, g3- and g4-HEV. Cyclic peptides corresponding to C15 of g1-HEV (APAHDTPPVPDVD) and an analogous sequence in g3-HEV (SPAPDTAPVPDVD) were synthesized. A linear epitope (QQDKGIAIPHDIDL), which was earlier characterized and known to generate neutralizing antibodies was used as a positive control (P6, 31). A chemiluminescent Enzyme-Linked Immunosorbent Assay (ELISA) was performed to quantify the reactivity of different peptides with reference and control sera. Six reference sera panels (PS1-PS6) and two healthy anti-HEV negative sera (HC-1, HC-2) were used in the assay (Table 1). All peptides showed

highest reactivity with PS1, which shows high anti-HEV IgG titer, followed by PS2 (Figure 2A). There was significant but relatively low reactivity of PS3-PS6 sera with the peptides (Figure 2A). To further substantiate the data, reactivity of C15 peptide was further tested using sera obtained from 33 acute HEV infected patients and 80 HEV exposed CLD patients and 6 healthy HEV negative sera (Table 1, Table S5). 23 of 26 HEV-AVH, 7 of 7 HEV-(ALF+ACLF) and 46 of 80 HEV-CLD sera showed significant reactivity with C15 peptide, further supporting the presence of antibodies against C15 epitope in HEV positive sera (Figure 2B).

Hecolin vaccine based on *E.coli*-expressed 368-606aa of the ORF2 protein is commercially available and known to offer protective immunity (24). Another HEV vaccine candidate based on Baculovirus-expressed 112-608aa of the ORF2 protein is also known to offer protective immunity (28). Since C15 epitope is present only in the 112-608aa ORF2 vaccine antigen, we compared the immunoreactivity of 368-606 and 112-608 ORF2 proteins to gain functional insight into the role of C15 epitope.

Purification and characterization of 368-606 and 112-608 ORF2 protein.

Recombinant VLPs containing 368-606 of g1-HEV ORF2 were purified from the insoluble fraction of *E.coli*, by two rounds of immobilized metal ion affinity chromatography (IMAC), followed by density gradient centrifugation (Figure S4A, S4B) (32). Specificity of the protein was confirmed by western blot analysis using anti-ORF2 antibody (Figure S4C). VLP nature of the protein was verified by transmission electron microscopy (Figure S4D). Dynamic light scattering (DLS) revealed the average hydrodynamic diameter of the VLP to be (20.4 ± 0.35) nm.

Earlier studies have shown that baculovirus expressed 112-608 ORF2 forms VLP and protects against HEV infection. However, Baculovirus expressed 112-608 ORF2 is glycosylated, which may influence its immunogenicity and virus neutralization potential. In order to rule out the influence of glycosylation, we attempted to purify the 112-608 ORF2 from *E.coli*. Expression of the 112-608 ORF2 in *E.coli* resulted in accumulation of the recombinant protein in the inclusion bodies (Figure S4E). This protein formed aggregates and precipitated upon storage. We reasoned that improving total hydrophilicity of the 112-608 ORF2 protein might enhance its solubility and facilitate VLP formation. Protein sequence corresponding to amino acid residues 112-608 of ORF2 of human-infecting HEV genotypes (g1: AF444002.1, g2: APG39846.1, g3: JQ679013.1, g4: BAD34959.1, g7: AHY61296.1) were retrieved from NCBI and aligned to generate the consensus sequence. The consensus sequence differs from g1-HEV sequence at 18aa residues (Figure S5A). The total hydrophilicity of each sequence calculated using Kyte/Doolittle method [KD] was found to be: 62.27, 65.07, 78.81, 75.90 and 43.10, for g1-, g2-, g3-, g4- and g7-HEV, respectively (33). Total KD hydrophilicity of the consensus sequence was found to be 62.21. Since total hydrophilicity of g3-ORF2 sequence was the highest among all, consensus sequence was manually scrutinized and specific

residues were altered in favour of g1 or g3-ORF2 sequence to improve its total hydrophilicity. The alteration of 10 residues in the consensus sequence [A3S, V77A, S153T, A315T, (S,A,T)/372A, (G,N,D)/379N, V381M, (S,P,T,M,K)/416T, V482A, G488A] increased its total hydrophilicity to 74.41. Next, 3D structure of g1-HEV 112-608 ORF2 protein sequence containing 21 substitution mutations (A3S, H6P, P9A, T38S, S50N, S153T, Y243H, S246G, I253V, A315T, S372A, G379N, V381M, S386T, L389F, T406S, S416T, L458I, V460I, V482A, V495A) was compared to that of the wild type protein to rule out any structural deviation. Wild type g1-HEV 112-608 ORF2 protein crystal structure was downloaded from the RCSB Protein data bank [PDB ID: 2ZZQ] (34). 3D structure of the g1-HEV 112-608 ORF2 and g1-HEV 112-608 ORF2-mutant (21aa) were predicted on the Alpha Fold server (35). Predicted structures were compared to the crystal structure using PyMOL align and super methods (36). G1-HEV 112-608 ORF2 protein crystal structure and Alpha fold generated structures were similar [RMSD: 0.567 (Align) and 0.553 (Super)] (Figure 3A). G1-HEV 112-608 ORF2 protein crystal structure and Alpha fold generated g1-HEV 112-608 ORF2-mutant protein (21aa) structures were also similar [RMSD: 0.546 Å (Align) and 0.528 Å (Super)] (Figure 3B). Further, Alpha fold generated g1-HEV 112-608 ORF2 protein (wild type) and Alpha fold generated g1-HEV 112-608 ORF2-mutant protein (21aa) structures were also similar [RMSD: 0.152 Å (Align) and 0.141 Å (Super)] (Figure 3B).

Codon optimized DNA sequence corresponding to the 112-608 ORF2-mutant protein (21aa) was synthesized for experimental verification of its solubility status in *E.coli* expression system. However, a V353G mutation was inadvertently introduced into the protein sequence during the synthesis process. This substitution mutation further increased the total hydrophilicity of the mutant protein to 79.01, without significantly altering its 3D structure, compared to the wild type protein [RMSD: 0.27 Å (Align) and 0.26 Å (Super)] [Figure 3B, g1-HEV 112-608 ORF2-mutant protein (22aa)]. Further, normal mode analysis of the wild type and g1-HEV 112-608 ORF2 mutant (22aa) proteins using DynaMut2 suggested a similar profile of both proteins, evident from their dynamic cross correlation map (DCCM) (Figure 3C, 3D) (37). Collectively, above data suggest that both wild type and g1-HEV 112-608 ORF2 mutant (22aa) proteins are similar in terms of their structural environment and protein dynamics. Expression of the g1-HEV 112-608 ORF2 mutant (22aa) protein resulted in partial solubilization (~50%) of the protein, which was purified by two rounds of IMAC (Figure S4E, S5B, S5C). Specificity of the protein was confirmed by western blot analysis using anti-ORF2 antibody (Figure 3E). VLP nature of the protein was verified by transmission electron microscopy (Figure 3F). DLS analysis revealed the average hydrodynamic diameter of the VLP to be (26.8±5.0)nm. Purified 112-608 ORF2 was further evaluated by western blot and ELISA analysis using the HEV positive and healthy HEV negative sera used in the cyclic peptide microarray. As expected, it was recognized by the HEV positive sera (Figure 3G, 3H).

C15 epitope contributes to superior immunogenicity and virus neutralization potential of the 112-608 ORF2 protein.

To compare the immunogenicity and virus neutralization potential of 112-608 ORF2 and 368-606 ORF2, BALB/c mice were intraperitoneally immunized with respective proteins (ALUM adjuvant formulation of 30 μ g purified antigen in 1:1 ratio). One prime dose and two booster doses were administered at 2 weeks interval, followed by collection of blood and spleen samples for analysis of different immune parameters (Figure 4A). Significant level of anti-ORF2 IgM was detected at 1 and 2 weeks of post-immunization in the sera of mice immunized with both 368-606 and 112-608 ORF2, the latter being significantly higher (Figure 4B). Measurement of anti-ORF2 IgG titre in the sera collected on week 4 and week 6 from 368-606 and 112-608 ORF2 immunized mice revealed the average values to be 1×10^4 and 3.2×10^4 (anti-ORF2-IgG titre at week 4) and 5×10^4 and 1.5×10^5 (anti-ORF2-IgG titre at week 6), respectively (Figure 4C). 50% virus neutralization titre (NT₅₀) in the sera collected on week 6 from 368-606 and 112-608 ORF2 immunized mice was estimated to be 0.8×10^3 and 1.0×10^4 , respectively (Figure 4D). HEV positive human reference sera (PS1) was used as a control to compare with the NT₅₀ of immunized mice sera. Significantly higher anti-ORF2-IgG titre and NT₅₀ was observed in 112-608 ORF2 immunized mice sera than that of the 368-606 immunized mice sera. Further, NT₅₀ of 112-608 ORF2 immunized mice sera was similar to that of the PS1 (Figure 4D). IgG-isotyping of aliquots of the sera collected on week 6 showed higher IgG1 and IgG2a in 112-608 ORF2 immunized mice (Figure 4E). The ratio of IgG2a to IgG1 suggested T_H1-biased response (Figure 4F). Next, splenocytes from the immunized mice were cultured *in vitro* and induced with overlapping ORF2 peptide pool, followed by selection of CD4⁺ and CD8⁺ T cells and quantification of intracellular T_H1 (IFN- γ , TNF- α) and T_H2 (IL-4) cytokines. Both CD4⁺ and CD8⁺ T cells of mice immunized with 368-606 and 112-608 ORF2 induced significant IFN- γ and TNF- α response (Figures 4G-4H). IFN- γ and TNF- α response in CD4⁺ T cells were higher in the 112-608 ORF2 immunized mice compared to that in the 368-606 ORF2 immunized mice (Figure 4G). However, there were no significant difference in IFN- γ and TNF- α response in CD8⁺ T cells in both 368-606 and 112-608 ORF2 immunized mice (Figure 4H). There were no significant changes in IL-4 response in both 368-606 and 112-608 ORF2 immunized mice, indicating that both vaccine formulations induced T_H1-biased T-cell immune response (Figures 4G, 4H). Gating strategy and representative FACS plots are shown (Figure S6, S7). Anti-ORF2 IgG titre and NT₅₀ in the sera collected after 1 year of first immunization from 368-606 and 112-608 ORF2 immunized mice was found to be 6×10^4 and 9×10^4 (anti-ORF2-IgG titre of 368-606 and 112-608 ORF2) and 1×10^3 and 5×10^3 (NT₅₀ of 368-606 and 112-608 ORF2), respectively (Figure 4I, 4J). Collectively, these data support superior immunogenicity and virus neutralization potential of 112-608 ORF2 over 368-606 ORF2.

To investigate the role of C15 epitope in mediating superior immunogenicity and virus neutralization potential of 112-608 ORF2, an immunodepletion assay was performed. Sera collected on week 6 from 112-608 ORF2 immunized mice were pooled and incubated with C15 peptide or 368-606 ORF2 protein, depleting corresponding antibodies, followed by ELISA of 500X-4500X diluted sera to measure their reactivity with the 112-608 ORF2 protein. Mock depletion and adjuvant immunized sera were used as negative controls. Five rounds of immunodepletion of 368-606 ORF2 immunized sera with 368-606 ORF2 protein completely depleted all corresponding antibodies, indicating that the assay behaved as expected (Figure 5A, 500X diluted immunodepleted sera). 1500X and 4500X diluted 368-606 ORF2 immunized sera immunodepleted with 368-606 ORF2 protein showed complete depletion of all corresponding antibodies in fourth and third round of immunodepletion, further supporting the above data (Figure 5B, 5C). At 500X dilution, 112-608 ORF2 immunized mice sera immunodepleted with C15 peptide showed reduced anti-ORF2 IgG level, compared to mock depleted sera (Figure 5A). 112-608 ORF2 immunized mice sera immunodepleted with 368-606 ORF2 protein retained significant level of anti-ORF2 IgG, supporting immunogenicity of peptides present outside the 368-606aa region of ORF2 (Figure 5A). Similar trend was observed in 1500X and 4500X diluted immunodepleted sera (Figure 5B, 5C). 112-608 ORF2 immunized mice sera immunodepleted for 7 rounds with the 368-606 ORF2 protein or C15 peptide showed 100 fold and 10 fold reduction in NT₅₀, suggesting that major neutralizing epitopes are present in the 368-606 ORF2 region, however, C15 epitope also significantly contributes to overall neutralization efficacy of the 112-608 ORF2 (Figure 5D). Results obtained in mice immunization study were corroborated with HEV positive human sera. ELISA of 150 HEV positive sera using equal amount of purified 368-606 ORF2 and 112-608 ORF2 proteins revealed higher reactivity of the later with the HEV positive reference sera (Figure 5E). Taken together, these findings support the role of C15 epitope in enhancing immunogenicity and virus neutralization potential of the 112-608 ORF2 protein.

Discussion

Detailed knowledge of immune epitopes is necessary for developing specific diagnostic assays, epidemiological monitoring, development of therapeutic antibody and vaccines. Induction of both B- and T-cell response is crucial for success of a vaccine. T-cell response is mediated by short linear (continuous) epitopes of 9-13aa whereas B-cell response is mediated by both linear (continuous) and conformational (discontinuous) epitopes. Earlier studies on HEV immune epitope mapping used linear peptides or purified recombinant proteins from different regions of the viral capsid protein (17-19). However, the proteome wide spectrum of HEV immune epitopes remain unknown. The current study focused on identifying the repertoire of B-cell epitopes present in human-infecting HEV genotypes.

Though both linear and cyclic peptides have been used to screen B-cell epitopes, cyclic peptides seem to be more efficient in reproducing native protein topology (38). Linear peptides represent continuous epitopes whereas cyclic peptides may display both continuous and discontinuous (conformational) epitopes. Compared to linear peptides, cyclic peptides are less flexible and retain a stable conformation due to the constraints imposed by cyclization. The constrained structure of cyclic peptides may partially reproduce native epitope topology (39, 40). Peptide display techniques such as phage display or microarray are reliable for screening the entire proteome for immune epitope discovery (41). By combining solid phase cyclic peptide synthesis with laser printing technology, high density linear or cyclic peptide microarrays have been produced (42). 11-15aa cyclic peptides have been used for different studies such as immune epitope discovery for vaccine development and antibody epitope mapping (43-47). In this study, 13aa cyclic peptides covering the proteome of g1-, g2-, g3- and g4-HEV (with 11aa overlap) were screened using a pool of HEV positive sera to discover all immunogenic epitopes (neutralizing and non-neutralizing) in an unbiased manner. 11aa overlap between two peptides ensured that most of the epitopes were reproduced. Though 13aa cyclic peptides may not be able to reproduce the conformation of large discontinuous epitopes, they mimic conformational epitopes by producing a defined 3D shape, exposing the loop structures on proteins.

Multiple controls and checks were included in the screening step to ensure that only specific signal was retained: (a) cyclic peptides corresponding to Influenza Hemagglutinin (HA), Polio virus and C-Myc epitopes were spotted on the same microarray slide containing HEV peptides (b) copy of the same microarray slides were stained with only secondary antibody to rule out non-specific signal(s) (c) different dilutions of the sera were assayed to select the optimal dilution (d) positive correlation between intensity plot and scanned images of the microarray was analyzed to verify reliability of the data.

Cyclic Peptide Microarray analysis revealed 21 unique immunogenic epitopes in g1-HEV, whereas 13, 14 and 9 epitopes were detected in g2, g3a and g4-HEV, respectively. Expectedly, maximum number of epitopes were detected in the ORF2 region, 4 of them being in the known major neutralization region (C17-C20). This finding supports the efficacy of the cyclic peptide microarray technique in identifying immunogenic epitopes in HEV. The epitopes identified in the ORF2 region may produce a mixture of neutralizing and non-neutralizing antibodies, the later acting as immune decoys to promote viral escape. Two epitopes (C9, C10) were identified in the ORF3 protein, which is known to associate with ORF2 as well as promote progeny virus release (12). However, whether ORF3 is a component of mature virus remain inconclusive. Evaluation of virus neutralization potential of C9 and C10 epitopes in naturally circulating viruses will clarify the role of ORF3 as a structural component of the virus. Eight epitopes were identified in the ORF1 region. Since ORF1 is synthesized from the

genomic RNA immediately after infection, characterization of ORF1 epitopes may identify biomarkers for HEV diagnosis.

We focused on functional characterization of the C15 epitope, primarily because (a) it showed higher reactivity to the human sera in the cyclic peptide microarray despite being located outside the known major neutralizing region and (b) this epitope is mostly conserved in all human-infecting HEV genotypes. C15 epitope of g1- and g3-HEV differs at three amino acid residues (A-S, H-P and P-A), whereas it differs at one residue between g1- and g2- HEV (P-S) and differs at 2 residues between g1- and g4- HEV (H-P, P-A). Given that maximum difference in C15 epitope was observed between g1- and g3-HEV, C15 peptides corresponding to g1- and g3-HEV were further evaluated using a panel of HEV IgM, IgG positive sera. Both linear and cyclic C15 peptides of g1- and g3-HEV showed a comparable reactivity with the reference sera, suggesting the epitope to be continuous. Further, sera from AVH category (2 weeks after infection, PS1 and PS2) showed highest reactivity with the C15 peptide whereas sera from CLD category showed much lower reactivity despite having high anti-HEV IgG titre (Compare PS2 to PS3 and PS5), indicating that antibodies corresponding to the C15 epitope wane faster. C15-epitope may be evaluated as a marker for differentiating between acute and chronic HEV infection. Further studies using more clinical samples is required to confirm the above proposition.

Preliminary data to indicate that C15 is a neutralizing epitope was derived by comparing the NT₅₀ titre of 112-608 and 368-606 ORF2 antigens. However, C21 epitope is also partially present in the 112-608 ORF2 antigen and its contribution towards enhancing the NT₅₀ of the 112-608 ORF2 antigen cannot be ruled out. Therefore, an immunodepletion followed by FRNT assay was performed using C15 peptide, which proved its ability to neutralize HEV.

Identification of a neutralizing epitope exclusively in the 112-608 ORF2 antigen has a significant impact on HEV vaccine development. At present, Hecolin is the only commercially available vaccine against HEV. It contains 368-606 ORF2 VLP reconstituted from the inclusion bodies of *E.coli* as the vaccine antigen. Earlier studies on HEV vaccine used Baculovirus expressed, glycosylated 112-608 ORF2 VLP as the vaccine antigen. In order to compare the immunogenicity and virus neutralization ability of 112-608 ORF2 with the 368-606 ORF2 VLP, we engineered the former to produce soluble VLPs (non-glycosylated) in *E.coli*. Note that recent reports have shown the production of both glycosylated and non-glycosylated ORF2-containing viruses during HEV infection, the latter being infectious (48). Use of *E.coli* expression system allows production of non-glycosylated 112-608 ORF2 VLP. Our study demonstrates superior and more durable immunogenicity, higher NT₅₀ titre and better T-cell memory response of the engineered 112-608 ORF2 VLP antigen. These findings pave the way for development of a HEV vaccine based on the *E.coli* produced 112-608 ORF2 VLP antigen.

In conclusion, the current study provides a comprehensive proteome wide map of B-cell epitopes present in human-infecting HEV genotypes. Functional characterization of one of the epitopes (C15) revealed it to be a neutralizing epitope, which contributes to superior immunogenicity and virus neutralization potential of the 112-608 ORF2 vaccine antigen. The C15 epitope may also be useful as a biomarker for HEV diagnosis. Further, we show that engineered 112-608 ORF2 protein produces soluble VLPs in *E.coli*, which acts as a more potent vaccine antigen than that used in the Hecolin vaccine. Functional characterization of the remaining epitopes may identify additional neutralizing epitopes and/or biomarkers of HEV.

Methods

Reagents and materials

Ni-NTA Superflow agarose resin (Catalog No. 25215) and NTA-Cobalt resin (Catalog No. 89964) were from Thermo scientific (Massachusetts, USA). Prolong gold antifade mountant with DAPI (4', 6-diamidino-2-phenylindole) (Catalog No. ab104139) was from Abcam (Cambridge, UK). The goat anti-Mouse IgG-HRP (Catalog No. 1030-05) and goat anti-human IgG-HRP (Catalog No. 2040-05) was from Southern Biotech (Alabama, USA). The goat anti-Rabbit IgG-HRP (Catalog No. 31460) were from Thermo Fisher Scientific (Massachusetts, USA). Anti-rabbit Alexa Fluor 594 was from Sigma-Aldrich (Missouri, USA). Anti-ORF2 antibody was developed in-house (13). PepMix™ HEV (ORF2) was from JPT peptide Technologies (Berlin, Germany). Cyclic and linear peptides were from PEPperPRINT (Heidelberg, Germany). Anti-mouse CD44 antibody (Catalog No. 103006), anti-mouse CD62L antibody (Catalog No.104445), anti-mouse CD3 antibody (Catalog No. 100229), anti-mouse CD8a antibody (Catalog No.100714), anti-mouse CD4 antibody (Catalog No.100552), anti-mouse IL-4 antibody (Catalog No. 504104), anti-mouse TNF- α antibody (Catalog No. 506324), anti-mouse IFN- γ antibody (Catalog No. 505810) were from BioLegend (California, USA).

Clinical sample collection and analysis, reference sera generation

Study population and design:

A total of 156 subjects were enrolled in this prospective cohort study conducted at two tertiary care hospitals: the Department of Gastroenterology, AIIMS, New Delhi, and the Department of Virology and Hepatology, PGIMER, Chandigarh. The study was carried out after obtaining approval from the Institutional Ethics Committee of AIIMS, New Delhi (approval no. IEC-612/03.09.2021.RP-33/2021) and PGIMER, Chandigarh (). The study participants were categorized into three cohorts: (a) the HEV-infected patient cohort (n = 48; AIIMS = 15, PGIMER = 33), comprising patients with active or recent HEV infection confirmed by the presence of HEV RNA and/or anti-HEV IgM antibodies; (b) the HEV-exposed patient cohort (AIIMS, n = 102), consisting of individuals with no evidence of active HEV infection but positive for anti-HEV IgG antibodies, indicating previous exposure; and (c) the healthy volunteer cohort (n = 6), including anti-HEV IgM and IgG seronegative individuals (Table

1). For the HEV-exposed cohort, consecutive patients with chronic liver disease (CLD) of viral (HBV and HCV) and non-viral etiologies (alcohol-associated liver disease, NASH, autoimmune liver disease, and cryptogenic liver disease) were prospectively screened for anti-HEV IgG antibodies, and those testing positive were included in the study.

Sample Collection from HEV infected patient cohort:

The blood samples at baseline were collected to detect acute HEV infection by anti-HEV IgM testing and/or HEV RNA testing for clinical diagnosis of AVH (acute viral hepatitis), ALF (acute liver failure) and ACLF (acute-on-chronic liver failure). The paired blood samples of anti-HEV IgM positive patients were further collected at hospital discharge (within 14 days) or at one month follow up for seroconversion to HEV and confirmed anti-HEV IgG positive samples were used in the study. Fecal samples were also collected from serum anti-HEV IgM and HEV RNA positive patients.

HEV-exposed patients and workup for Underlying CLD:

The CLD patients were diagnosed based on clinical evaluation, biochemical and serological tests, and radio-imaging studies. Blood samples were collected at single time point for screening of anti-HEV IgG and the positive patient sera were used in this study for validation experiment.

Serological Screening for HEV:

Serological testing for HEV at AIIMS, New Delhi was carried out by ELFA (enzyme linked immunofluorescence assay) using VIDAS Anti-HEV IgM and Anti-HEV IgG kit (Biomérieux, France), whereas at PGIMER, screening for anti-HEV IgM and IgG was carried out by ELISA using commercial kit (DiaPro, Italy). Anti-HEV IgM positivity was determined qualitatively when test index value is ≥ 1 , whereas Anti-HEV IgG positivity determined quantitatively and test value ≥ 0.56 U/mL was considered positive.

HEV RNA detection in clinical samples:

The HEV RNA detection for acute HEV infection was carried out quantitatively by real time PCR using RealStar HEV RT-PCR Kit 2.0 (Altona Diagnostics GmbH, Germany).

Generation of reference sera panels:

Six reference sera panels were generated by pooling the sera of HEV-infected and HEV-exposed patient cohort (categorized on the basis of quantitative determination of anti-HEV IgG titer). The reference sera PS1 (mean anti-HEV IgG Titer, 79.75 U/mL) and PS2 (5.42 U/mL) were collected from HEV infected patients seroconverted from acute HEV infection; reference sera PS3 (31.7 U/mL) and PS4 (9.34 U/mL) were collected from HEV exposed HBV-CLD patients; reference sera PS5 (17.54 U/mL) and PS6 (5.16 U/mL) were collected from HEV exposed: non-viral-CLD patients (Table S4).

Cyclic Peptide Microarray assay and data analysis

Three clinically confirmed HEV positive sera (#31390, #57527, #56797, see details in Table 1) and three healthy volunteer sera (#HC-1, #HC-2, #HC-3) were pooled and denoted as pooled HEV patient sera and pooled Healthy HEV negative sera, respectively. These sera were used in cyclic peptide microarray [following manufacturer's protocol, PEPperPRINT (Heidelberg, Germany)]. Overall assay protocol is summarized below. HEV peptide microarray was incubated for 15 min in washing buffer (PBS, pH 7.4 with 0.005% Tween 20), followed by 30 min incubation in blocking buffer (Rockland blocking buffer MB-070). Next, slide was incubated with secondary and control antibodies in incubation buffer (PBS pH 7.4, 0.005% Tween 20 and 10% Rockland blocking buffer MB-070) for 45 min at room temperature (RT) to analyze background interactions with the antigen-derived peptides that could interfere with the main assays. Read-out was carried out at scanning intensities of 7/7 (red/green). At scanning intensities of 7/7 (red/green), there was no background interaction of the secondary antibody with the cyclic constrained peptides even upon significant increase of brightness and contrast (Figure S1). Some very weak background interactions of the control antibody were observed with highly basic peptides like QAQRPAVASRRRP, QSQRPTAAPRRRP or AGQPSGRRRGRRS presumably due to less specific ionic binding and few cross-reactions with peptides like HSALAVLEDTADY or HSALAILEDTVDY due to sequence homology with the HA control peptides. However, these background interactions did not interfere with the analysis of the main assays in the red channel. Next, another set of slides were incubated with the human serum samples at dilutions of 1:1000 and 1:500 in incubation buffer (Washing buffer with 10% blocking buffer), followed by staining with secondary and control antibodies. The additional HA control peptides framing the peptide microarrays were simultaneously stained as internal quality control to confirm the assay quality and peptide microarray integrity. Quantification of spot intensities and peptide annotation were based on the 16-bit gray scale tiff files that exhibit a higher dynamic range than the 24-bit colorized tiff files. Microarray image analysis was done with PepSlide analyzer wherein a software algorithm breaks down fluorescence intensities of each spot into raw, foreground and background signal, and calculates averaged median foreground intensities and spot-to-spot deviations of spot duplicates. Based on averaged median foreground intensities, an intensity map was generated and interactions in the peptide map was highlighted by an intensity color code with red for high and white for low spot intensities. Maximum spot-to-spot deviation of 40% was tolerated, otherwise the corresponding intensity value was zeroed.

Bioinformatics analysis

Following GenBank accession numbers were used to download the protein sequences corresponding to residues 112-608aa of HEV ORF2: g1-HEV- AF444002.1, g2-HEV- APG39846.1, g3-HEV- JQ679013.1, g4-HEV-BAD34959.1, g7-HEV- AHY61296.1. Protein sequences were aligned in MacVector using ClustalW to generate the consensus sequence. The total hydrophilicity of each sequence was calculated in MacVector using Kyte/Doolittle method (33). Wild type g1-HEV 112-608 ORF2 protein crystal structure was downloaded from the RCSB Protein data bank (PDB ID: 2ZZQ) (34). 3D structure of the g1-HEV112-608 ORF2 and g1-HEV 112-608 ORF2-mutant

(21aa) was predicted on the AlphaFold server (35). Predicted structures were compared to the crystal structure using PyMOL align and super methods (Schrödinger LLC) (36). Predicted structures with RMSD < 0.7 Å were considered to be identical. Normal mode analysis of the wild type and mutant g1-HEV 112-608 ORF2 proteins were performed using DynaMut2 (37).

Purification of 112-608 and 368-606 ORF2 proteins

The g1-HEV 112-608 ORF2 sequence was PCR amplified from pSK-HEV-2 plasmid (GenBank accession number AF444002.1) using Primers (FP: GACCATATGGCGGTCGCTCCGGC and RP: AGTGCTCGAGTCACAGAGTG). PCR product was digested with NdeI and XhoI restriction enzymes and ligated into the pET 28a vector digested with the same. The g1-HEV 112-608 ORF2 mutant was commercially synthesized with flanking NdeI and XhoI restriction sites and cloned into the pUC57 vector (GenScript). pUC57 g1-HEV 112-608 ORF2 mutant was digested with NdeI and XhoI restriction enzymes and ligated into pET 28a vector digested with the same. Both clones were verified by restriction mapping and DNA sequencing. pET 28a-g1-HEV 112-608 ORF2 (wild type) and pET 28a-g1-HEV 112-608 ORF2 (mutant) were transformed into C41(DE3) competent cells. Single colony of each clone was inoculated in Luria Broth (LB) supplemented with 50 µg/mL Kanamycin and grown overnight (around 16 h) at 37 °C, 200 rpm, in a shaker-incubator. 1% of the overnight grown culture was inoculated in 250 mL LB + Kanamycin media and grown at 37°C, 200 rpm, in a shaker-incubator until A₆₀₀ reaches between 0.6-0.8. Expression of the 112-608 ORF2 protein was induced by addition of 0.2 mM IPTG to the culture medium, followed by incubation at 22 °C for 6 h. An aliquot of 1 mL of induced cells were centrifuged at 10,000 g for 10 min, washed once in PBS, cell pellet was resuspended in lysis buffer (20mM Tris-Cl pH 8.0, 150mM NaCl, 1mM PMSF, 10% Glycerol) and sonicated for 5 cycles (10'' on, 30'' off, 30% power rate) in an ultrasonic homogenizer, followed by centrifugation at 10,000 g for 10 min to separate soluble and insoluble fractions. Expression of the 112-608 ORF2 protein in soluble fraction was observed by Coomassie Brilliant Blue (CBB) staining of SDS-gel. For large scale culture, the induced culture media was harvested, washed in PBS, resuspended in lysis buffer, followed by separation of soluble and insoluble fractions using a high pressure Homogenizer (GEA Niro Soavi Homogenizer PandaPLUS 2000). Homogenized samples were centrifuged at 7800 g for 1 h and supernatant was collected. Collected supernatant was incubated with Ni²⁺-NTA Agarose beads for 2 h. Beads were washed with 5 CV (Column Volume) of wash buffer (20 mM Tris-Cl pH 8.0, 150 mM NaCl, 25 mM imidazole) and bound proteins were eluted in 5 CV of 100 mM-500 mM imidazole gradient. Aliquots of samples were analysed on SDS-PAGE by CBB staining and fractions containing 112-608 ORF2 protein were pooled for further purification. The Pooled fraction from Ni²⁺-NTA immobilized Metal affinity chromatography (IMAC) was buffer exchanged with equilibration buffer (20 mM Tris-Cl pH 8.0, 150 mM NaCl) and incubated with NTA-Cobalt-Dynabeads for 2 h, washed with 5 CV of wash buffer. Bound proteins were eluted with 5 CV of 100 mM-500 mM imidazole gradient. Eluted samples were analysed by SDS-PAGE, CBB staining and fractions containing 112-608 protein were pooled, concentrated and buffer exchanged to PBS using a 30 kDa centrifugal filter device (Pall Corporation,

New York, United States). Glycerol was added to a final concentration of 10% and protein was stored in aliquots at -80°C.

The g1-HEV 368-606 ORF2 was PCR amplified from pSK-HEV-2 plasmid (GenBank accession number AF444002.1) using Primers (FP: GACCATATGGCGGTCGCTCCGGC and RP: AGTGCTCGAGTCACAGAGTG). The PCR product was digested with NdeI and XhoI restriction enzymes and ligated into pET 28a vector digested with the same. pET28a-g1-HEV 368-606 ORF2 was transformed into C41(DE3) competent cells. Single colony was inoculated in Luria Broth (LB) supplemented with 50 µg/mL Kanamycin and grown overnight (around 16 h) at 37 °C, 200 rpm, in a shaker-incubator. 1% of the overnight grown culture was inoculated in 250mL LB+Kanamycin media and grown at 37 °C, 200 rpm, in a shaker-incubator until A₆₀₀ reached between 0.6-0.8. Expression of the g1-HEV 368-606 ORF2 was induced by addition of 0.5 mM IPTG to the culture medium, followed by incubation at 37 °C for 6 h. Protein expression was verified as described above using 1 mL induced culture. Large scale culture was processed as described above and pellet containing the inclusion bodies was treated 2M-4M Urea gradient for solubilizing the proteins. Samples were centrifuged at 7800 g for 1 h and supernatant was collected. Supernatant was buffer exchanged with equilibration buffer containing 2M Urea (20mM Tris-Cl, 150mM NaCl, 1mM PMSF, 10% glycerol, 2M Urea). Resulting samples were buffer exchanged with equilibration buffer (20mM Tris-Cl, 150mM NaCl, 1mM PMSF, 10% glycerol). Supernatant was incubated with Ni²⁺-NTA Agarose beads for 2 h. Beads were washed 5 CV wash buffer (20mM Tris-Cl pH 8.0, 150 mM NaCl) containing 25 mM imidazole and 50 mM imidazole, respectively. Bound proteins were eluted in 5 CV of 100 mM-300 mM imidazole gradient. Aliquots of samples were analysed by SDS-PAGE, CBB staining and fractions containing 368-606 protein were pooled. Pooled protein sample was laid over 10-40% density gradient of OptiPrep prepared in 1X TEN buffer (20mM Tris-Cl pH 8.0, 1mM EDTA, 150mM NaCl) and centrifuged at 285,000 g for 16 h at 4°C in a SW 45 Ti Rotor (Beckman, California, USA). Samples were collected in 1 mL aliquots from top and analysed by SDS-PAGE, CBB staining. Fractions with 368-606 ORF2 protein were pooled, concentrated and buffer exchanged to PBS using a 10 kDa centrifugal filter device. Glycerol was added to a final concentration of 10% and protein was stored in aliquots at -80 °C.

Mice Experiments

The mice experiment protocol was approved by the Animal Ethics Committee of Translational Health Science and Technology Institute (THSTI), constituted under the provision of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), Government of India.

Five groups of 6-8 week old male/female mice (n=6/group) were used for the study. Two groups were immunized with either 30µg of 112-608 ORF2 VLP or 368-606 ORF2 VLP emulsified with ALUM (1:1 volumetric ratio) by intraperitoneal route. Mice were administered with booster doses twice with 30µg immunogens at 2 weeks interval. Control group was administered with ALUM

formulated with PBS (1:1 ratio). Blood samples were collected before each immunization, sera was separated and stored at -80 °C.

ELISA and Chemiluminescence ELISA

ELISA was done for purified protein antigen-coated plates as described (49). 250 ng purified protein was coated in each well. Chemiluminescence ELISA was performed for Peptide antigen-coated plates. The biotinylated C15 peptide of g1-HEV (APAHDTTPVPDVD) and C10 peptide of g3-HEV (SPAPDTAPVPDVD) were synthesized in both cyclic and linear forms (PEPPERPRINT, Heidelberg, Germany). Peptides were dissolved to a working concentration of 10 µg/mL in coating buffer (PBS, pH 7.4 with 0.05% Tween 20 and 10% Rockland MB-070). 50 µL/well of 10 µg/mL peptide was coated in 96 well streptavidin coated plates and incubated for 16 h at 4 °C. Next day, wells were washed three times for 1 min each with PBST (PBS + 0.05% Tween 20) and 100 µL of serum sample (1:200 dilution) in incubation buffer (PBS, pH 7.4 with 0.05% Tween 20 and 10% Rockland MB-070) was added to each well. Plate was incubated overnight at 4 °C. Next day, wells were washed 3 times with PBST for 1 min each, 100 µL of Peroxidase (POD) labelled goat anti-human IgG secondary antibody (1:10,000 dilution) in incubation buffer was added and plate was incubated for 1 h at 20 °C. Wells were washed three times for 1 min each with PBST, 100 µL of BM-Chemiluminescence ELISA substrate (Sigma-Aldrich, Missouri, USA) was added and luminescence was measured at 460/40_{nm}.

Flow cytometry

Flow cytometry was done as described (50). Immunized mice were euthanized in CO₂ chamber and spleen was collected from the euthanized mice in 1 mL complete RPMI media (RPMI + 10% FBS + 1% penicillin-streptomycin antibiotic). Spleen was manually homogenized, passed through 70 mm cell-strainer and was collected in 15 mL conical tubes. The cells were centrifuged at 280 g for 10 min at 4 °C. Cell pellet was washed with complete RPMI media and resuspended in 2 mL ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA), mixed well and was incubated for 1 min at RT. 4 mL complete RPMI media was added to the cells to neutralize the lysis buffer. Samples were centrifuged at 280 g for 10 min at 4 °C. The cell pellet was resuspended in 2 mL of complete RPMI media and viable cells were counted by trypan blue staining method.

10⁶ cells/well splenocytes were seeded in 96-well plate. After 3 h, the ORF2 peptide pool were added (0.2 µg/mL/well) and cells were incubated for 4 h in a CO₂ (5%) incubator at 37 °C. Next, Golgi stop (BD) was added to the cells and incubated further for 12 h. Unstimulated cells were maintained in parallel. Next, cells were pelleted by centrifugation at 280 g for 10 min, washed with PBS and resuspended in 100 µL FC-block for 15 min at RT. Cells were stained with anti-CD16/32 and live dead stain for 30 min each at 4 °C and then stained for extracellular markers like anti-CD3, anti-CD4, anti-CD8 and anti-CD44 for 45 min at 4 °C. Cells were then fixed and permeabilized for intracellular cytokine (IFN γ , IL4 and TNF α) staining with respective antibodies for 1 h at 4 °C. Cells were sorted and analysed using BD FACS symphony A5SE.

Purification and quantification of infectious HEV from a clinical isolate

Faecal sample was obtained from an acute HEV patient (with informed consent), admitted to the Gastroenterology department of All India Institute of Medical Sciences, New Delhi, India, following the institutional guidelines. The infectious HEV from clinical isolates was purified as described (31). Briefly, the faecal sample was resuspended in PBS at 10% final concentration (w/v) and centrifuged at 7800 g for 1 h at 4 °C. The collected supernatant was passed through a 0.45 µm filter and 8% PEG6000 prepared in 0.4 M NaCl was added to the virus supernatant, followed by incubation for 16 h at 0 °C. The sample was centrifuged at 18,000 g for 30 min at 4 °C. The supernatant was removed and pellet was resuspended in 40 mL of Dulbecco's Modified Eagle Medium (DMEM). Concentrated the suspension to 5 mL volume by tangential flow filtration (TFF) using a 30 kDa membrane cartridge. Resulting viral suspension was filtered through a 0.2 µm filter inside the Biosafety cabinet, aliquoted, and stored at -80 °C. One aliquot was used to quantify the HEV genomic RNA level by RT-qPCR using the following primers: g1-HEV FP: 5' TATACTCGAGGGTGCCGATCGGTCCC 3'; g1-HEV RP: 5' TATACCATGGCATCTGGCAGCAAGCTCAG 3'; g1-HEV Probe: 5'[FAM] TTGACGCCTGGGAGCGGAATCACC [BHQ1]3'. The genome copy number was calculated using the following formula:

Number of copies = $\text{ng} \times (\text{number/mole}) / \text{bases} \times (\text{ng/g}) \times (\text{g/mole of bases})$.

Virus neutralization Assay

Virus neutralization assay was performed as described (31). Briefly, 0.2×10^6 Huh7 cells/well were seeded on coverslips placed in 12-well plate. The 10-fold serial dilution of serum samples (1:10 up to 1:10⁵ dilution) was prepared in DMEM. Purified g1-HEV (equivalent to 1.8×10^4 genomic copies) was added to each serum dilution. The antibody and virus mixture was incubated in a 5% CO₂ incubator, 37 °C for 1 h. Culture media was removed from cells in 12-well plate and cells were washed with PBS. The antibody-virus mixture was added to the cells and were incubated in a 5% CO₂ incubator at 37 °C for 1 h. Next, cells were washed with PBS and incubated in DMEM + 2% FBS media for 72 h, in a 5% CO₂ incubator at 37 °C.

After 72 h of infection, cells were washed three times with PBS, for 5 min each, at room temp (RT) and fixed with 4% Paraformaldehyde (PFA) for 20 min at RT. Cells were washed three times with PBS, followed by incubation in PBS for 30 min. Blocking solution (5% Normal Goat Serum, 0.5% BSA, 0.3% Triton X-100 in PBS) was added to the cells and incubated for 1 h at RT. Next, cells were incubated with primary antibody (PBS, 0.3% Triton X-100, 5% BSA, 0.5% normal goat serum, 1:500 dilution of anti-ORF2 antibody), for overnight at 4 °C. Next day, cells were washed three times with PBS, for 5 min each, incubated with secondary antibody (PBS, 0.3% Triton X-100, 5% BSA, 0.5% normal goat serum, 1:1000 dilution of goat anti-rabbit Alexa fluor 594 antibody) for 1 h at RT and washed three times with PBS, for 5 min each. A drop of prolong gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole) solution was added to the glass slide, coverslip-containing

cells were laid over it (with the cells facing towards the DAPI solution on glass slides), avoiding air bubbles and contact surface was sealed using nail paint. Images were captured at 10X magnification in a confocal microscope (Olympus FV3000) and analysed using OLYMPUS FLUOVIEW software. Fluorescent spots were quantified using image J, and percentage (%) neutralization of each dilution was calculated using the following formula:

% Neutralization =

$$100 - [(ORF2\text{-Positive cells in dilution}) / (ORF2\text{-positive cells in virus control}) \times 100]$$

Further, NT₅₀ was calculated using the formula mentioned below:

$$\text{Log}_{10}(\text{NT}_{50}) = \text{Log}_{10} D1 + (50 - N1) / (N2 - N1) \times (\text{Log}_{10} D2 - \text{Log}_{10} D1),$$

$$\text{NT}_{50} = 10^{\text{Log}_{10}(\text{NT}_{50})}$$

Immunodepletion assay

500 µL dynabeads MyOne tosylactivated (Thermo Fisher Scientific, Massachusetts, USA) was washed once in 1 mL of coating buffer (0.1M Sodium Borate, pH 9.5), resuspended in 100 µL coating buffer and 635 µL purified N-protein (2 mg of protein). 415 µL of 3 M Ammonium Sulphate was added to the mix and incubated for 16 h at 37 °C on a rotator. Next day, the tube was placed on the magnetic stand, beads were collected and supernatant was discarded. 1 mL blocking buffer (PBS pH 7.4, 0.5% BSA and 0.05% Tween20) was added to the beads and incubated overnight at 37 °C on a rotator. Next day, supernatant was discarded, beads were washed three times in 1 mL of Washing/Storage Buffer (PBS pH 7.4, 0.1% BSA and 0.05% Tween20) and resuspended in 700 µL storage buffer. The beads were distributed in 7 microcentrifuge tubes, 100 µL each. Mice sera was diluted (1:5 dilution) in PBS to a total volume of 500 µL, added to the microcentrifuge tubes containing 100 µL coated beads and incubated on a rotator at 37 °C, 1 h. After 1 h, beads and supernatant were collected, 20 µL supernatant was stored as sample after depletion round one and rest of the supernatant was incubated with another tube of coated beads. Above steps were repeated for seven rounds of depletion. Samples were analysed by ELISA.

Statistical analysis

For mouse study, 6 animals were used in each group, except for 112-608 ORF2 immunized group in Figure 4I, where 4 animals survived after 1 year of immunization. Average values are shown in the bar and individual values are shown as dots in each bar in the graphs. In vitro assays were performed in duplicate or triplicate, as indicated in the respective figures. SD values were calculated only for triplicate data. Two-tailed paired student t-test was used for calculating the significance. Logistic regression was used to determine the NT 50 titre of the mice sera. All statistical analysis was performed using the GraphPad Prism 9. A p-value <0.05 with 95% confidence interval was considered statistically significant. ns: p>0.05, *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001.

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Author contribution

Conceptualization: MS, BN, RKR; Investigation: SA, JG, UB, GA, KC, AY, TRA, MFS, SP, PS; Data analysis: SA, JG, KC, GA, UB, AY, TRA, RKR, ST, KS, BN, S, SR, MFS, SP, PS, PG, KPG, MS; Manuscript writing: SA, BN, MS. All authors have reviewed, edited and agreed to the final version of the manuscript.

Declaration of interest

A patent application has been filed by MS and JG titled “Mutant HEV pORF2 based antigens and uses thereof”.

Keywords

Hepatitis E Virus; HEV vaccine; Virus-like particle vaccine; HEV Capsid; Neutralizing epitope; cyclic peptide microarray

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Figure 1

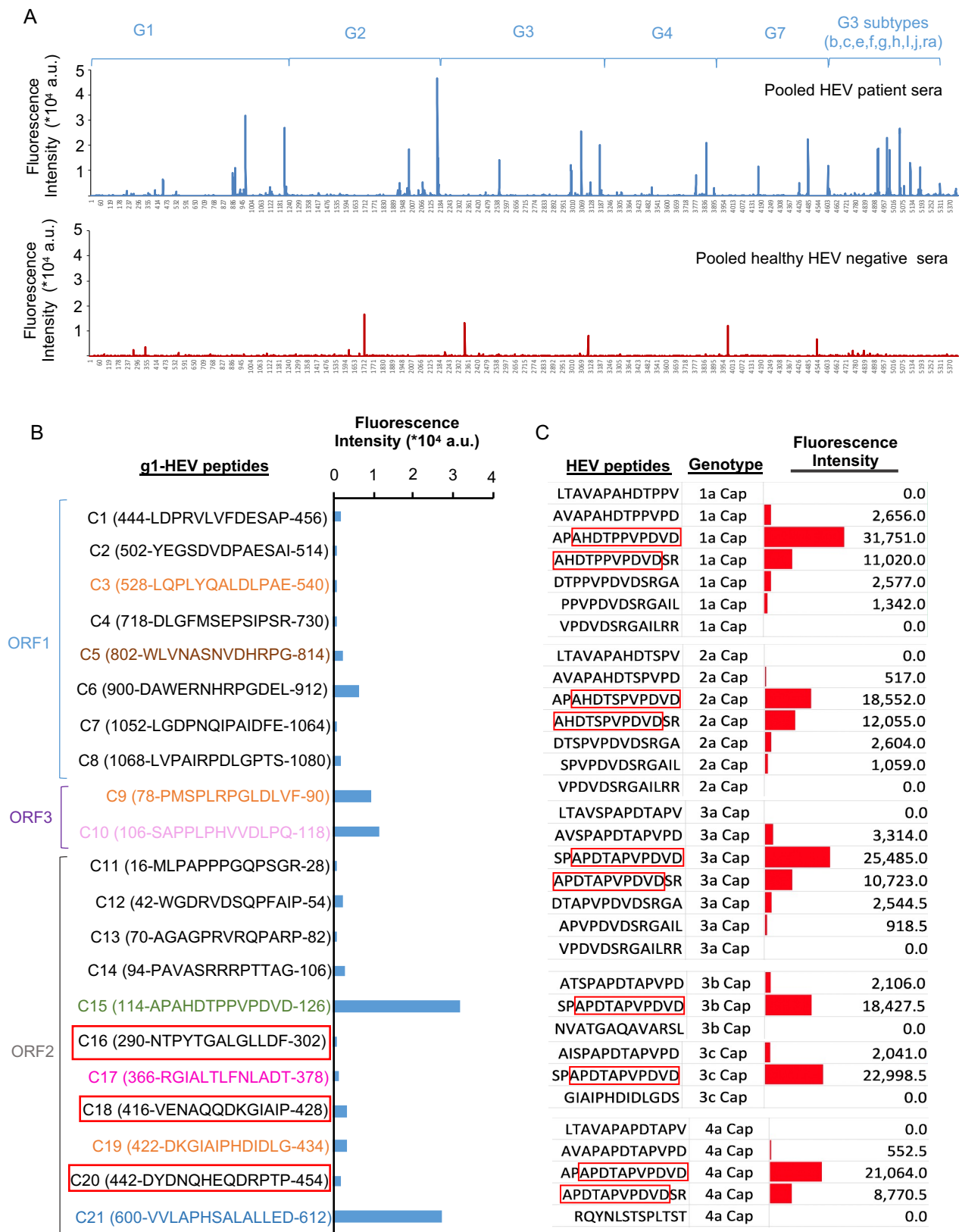


Figure 1: Cyclic peptide microarray of HEV patient sera reveals the repertoire of B-cell epitopes in HEV proteome.

A) Upper panel: HEV patient sera-microarray fluorescence intensity plot of cyclic peptides corresponding to g1-, g2-, g3-, g4- and g7-HEV proteomes. Lower Panel: healthy HEV negative volunteer sera-microarray fluorescence intensity plot of cyclic peptides corresponding to g1-, g2-, g3-, g4- and g7-HEV proteomes. Peptides with fluorescence intensity more than 500, were plotted. B) Peptide sequences and intensity plot graph of B-cell epitopes present in the g1-HEV ORF1, ORF2 and ORF3 regions. C) Peptide sequences and intensity plot graph of B-cell epitopes present in the ORF2 region of g1-, g2-, g3- and g4-HEV. Color coding: Red Box denotes earlier reported epitopes; Orange denotes epitopes common in g1- and g2-; Brown denotes common epitopes in g1-, g2- and g4-HEV; Lavender denotes common epitopes in g1-, g2-, g3a- and g4-HEV; Pink denotes common epitopes in g1-, g2- and g3-HEV; Green denotes g1-, g2-, g3a-, g3b-, g3c- and g4-HEV; Blue denotes common epitopes in g1-, g2-, g3a-, g3b-, and g3c-HEV; Purple denotes common epitopes in g3a- and g4-HEV.

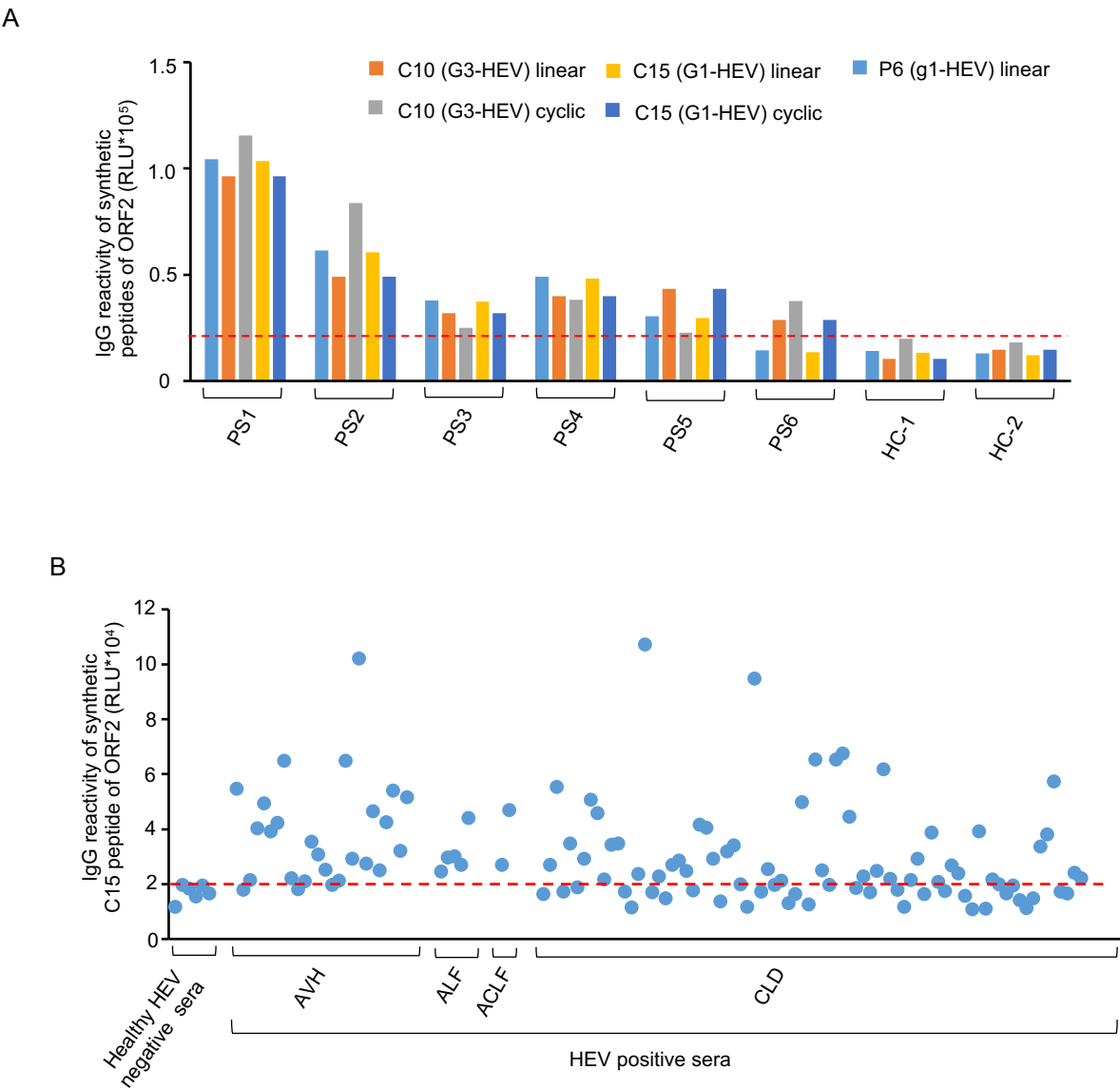


Figure 2: Reactivity of HEV peptides with HEV patient serum.

A) Peptide ELISA of pooled HEV patient sera (PS1-PS6) and healthy HEV negative volunteer (n=2) sera with synthetic peptides P6, C10 (g3-HEV) linear, C10 (g3-HEV) cyclic, C15 (g1-HEV) linear and C15 (g1-HEV) cyclic. ELISA was done in duplicate and average values were plotted. B) Scatter plot of cyclic peptide ELISA of HEV positive sera and healthy HEV negative volunteer sera with synthetic C15 peptide (n=113, HEV positive sera; n=6, healthy HEV negative volunteer sera).

Figure 3

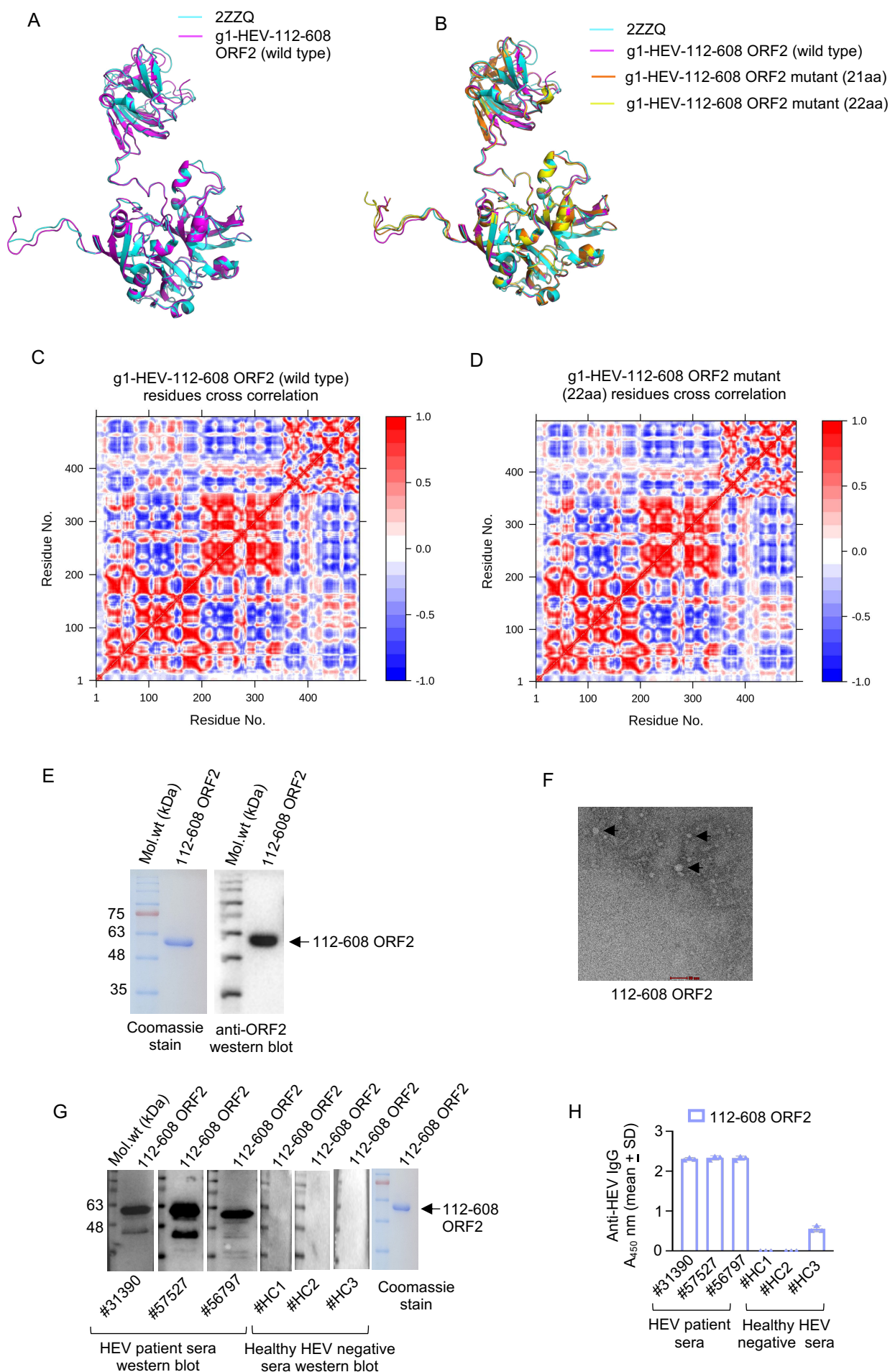


Figure 3: Alignment of 3D structure of wild type and mutant g1-HEV 112-608 ORF2, Purification and characterization of 112-608 ORF2.

A) Superimposed image of g1-HEV 112-608 ORF2 protein crystal structure (2ZZQ) with Alpha fold generated g1-HEV 112-608 ORF2 protein (wild type) using PyMOL Align and Super method. B) Superimposed images of g1-HEV 112-608 ORF2 protein crystal structure (2ZZQ) with Alpha fold generated g1-HEV 112-608 ORF2 protein (wild type), Alpha fold generated g1-HEV 112-608 ORF2-mutant proteins (21aa and 22aa) structures using PyMOL Align and Super method. C) Dynamic cross correlation map (DCCM) of g1-HEV 112-608 ORF2 (Wild type) using DynaMut2. D) Dynamic cross correlation map (DCCM) of g1-HEV 112-608 ORF2 mutant (22aa) using DynaMut2. E) CBB staining and western blot of purified 112-608 ORF2 with anti-ORF2 antibody. F) Transmission electron micrograph of purified 112-608 ORF2 protein (Scale: 50nm). G) Western blot of 112-608 ORF2 protein with HEV patient sera and healthy individual sera. CBB stained image is same as shown in (E). H) ELISA of HEV patient sera and healthy individual's sera with 112-608 ORF2. ELISA was performed in triplicate (n=3) and values are plotted as mean $\pm$ SD.

Figure 4

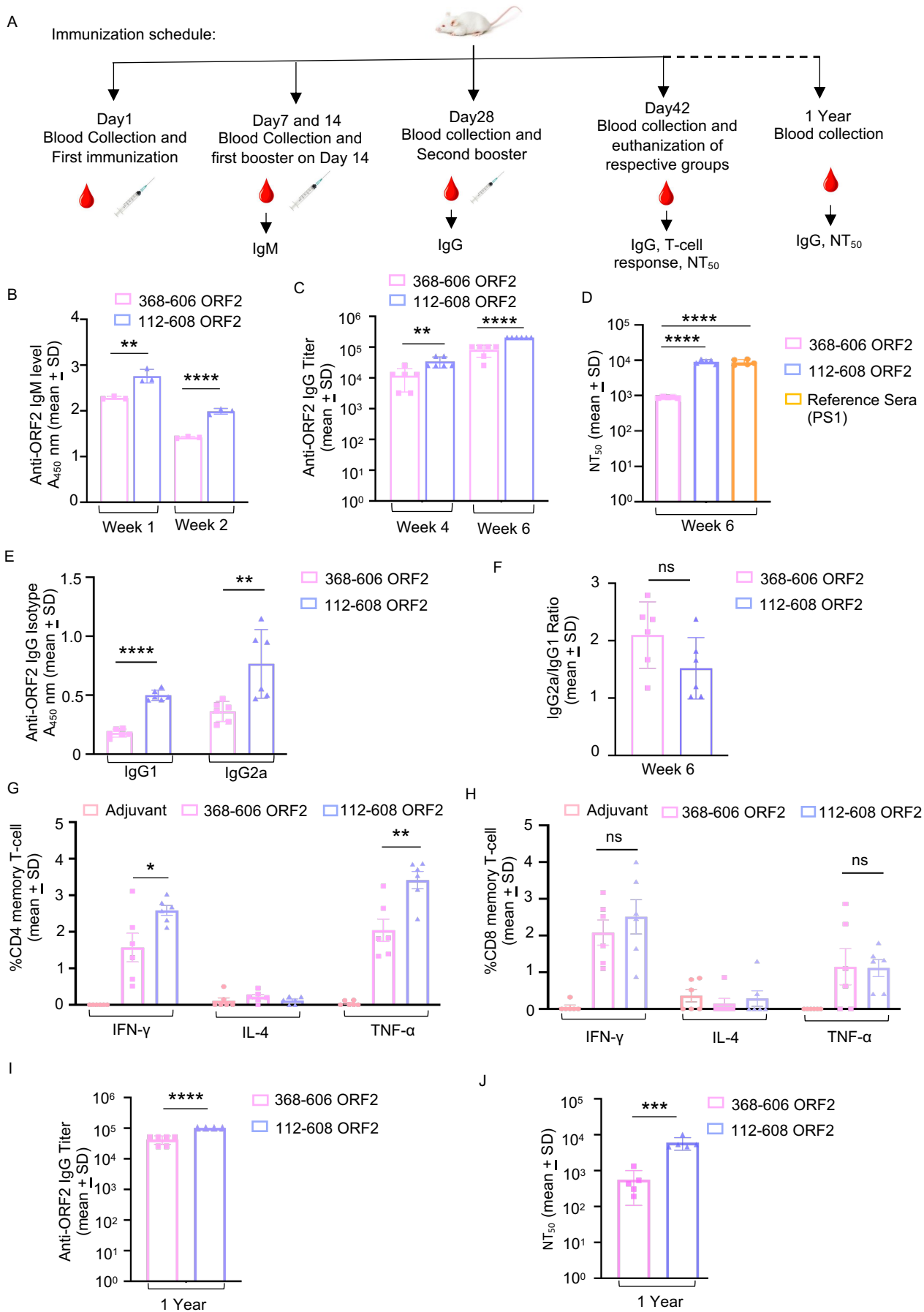


Figure 4: Evaluation of immunogenicity and virus neutralization potential of the purified 112-608 ORF2 and 368-606 ORF2 proteins.

A) Schematic of mice immunization schedule. B) Anti-ORF2 specific IgM response in 112-608 ORF2 and 368-606 ORF2 immunized mice sera on Day 7 and Day 14. Sera of all mice in a group were pooled and assayed in triplicate (n=3). C) Anti-ORF2 specific IgG antibody titer profile of 112-608 ORF2 and 368-606 ORF2 immunized mice sera on week 4, and week 6 (n=6). Titer is represented as Log₁₀ Value. D) NT₅₀ of 368-606 ORF2 and 112-608 ORF2 immunized mice sera and HEV convalescent human sera. E) Anti-ORF2 IgG isotyping of 112-608 VLP and 368-606 VLP immunized mice sera on week 4 (n=6). F) IgG2a/IgG1 ratio of 112-608 VLP and 368-606 VLP immunized mice sera on week 4 (n=6). G) Percent of CD4+ memory T-cells producing IFN- γ , TNF- α or IL-4. H) Percent of CD8+ memory T-cells producing IFN- γ , TNF- α or IL-4. I) Anti-ORF2 IgG antibody titer of 112-608 ORF2 and 368-606 ORF2 immunized mice sera upon 1 year of first immunization (n=6, 368-606 VLP immunized mice; n=4, 112-608 VLP immunized mice). Titer is represented as Log₁₀ Value. J) NT₅₀ of 368-606 ORF2 and 112-608 ORF2 immunized mice sera upon 1 year of first immunization. Serum neutralization was done by pooled sera of respective 368-606 ORF2 and 112-608 ORF2 immunized mice. Five fields (n=5) fields were analyzed to calculate NT₅₀. Data is represented as mean $\pm$ SD and P-value were calculated using student t-test. ns: P > 0.05, (*): P $\leq$ 0.05, (**): P $\leq$ 0.01, (***): P $\leq$ 0.001, (****): P $\leq$ 0.0001.

Figure 5

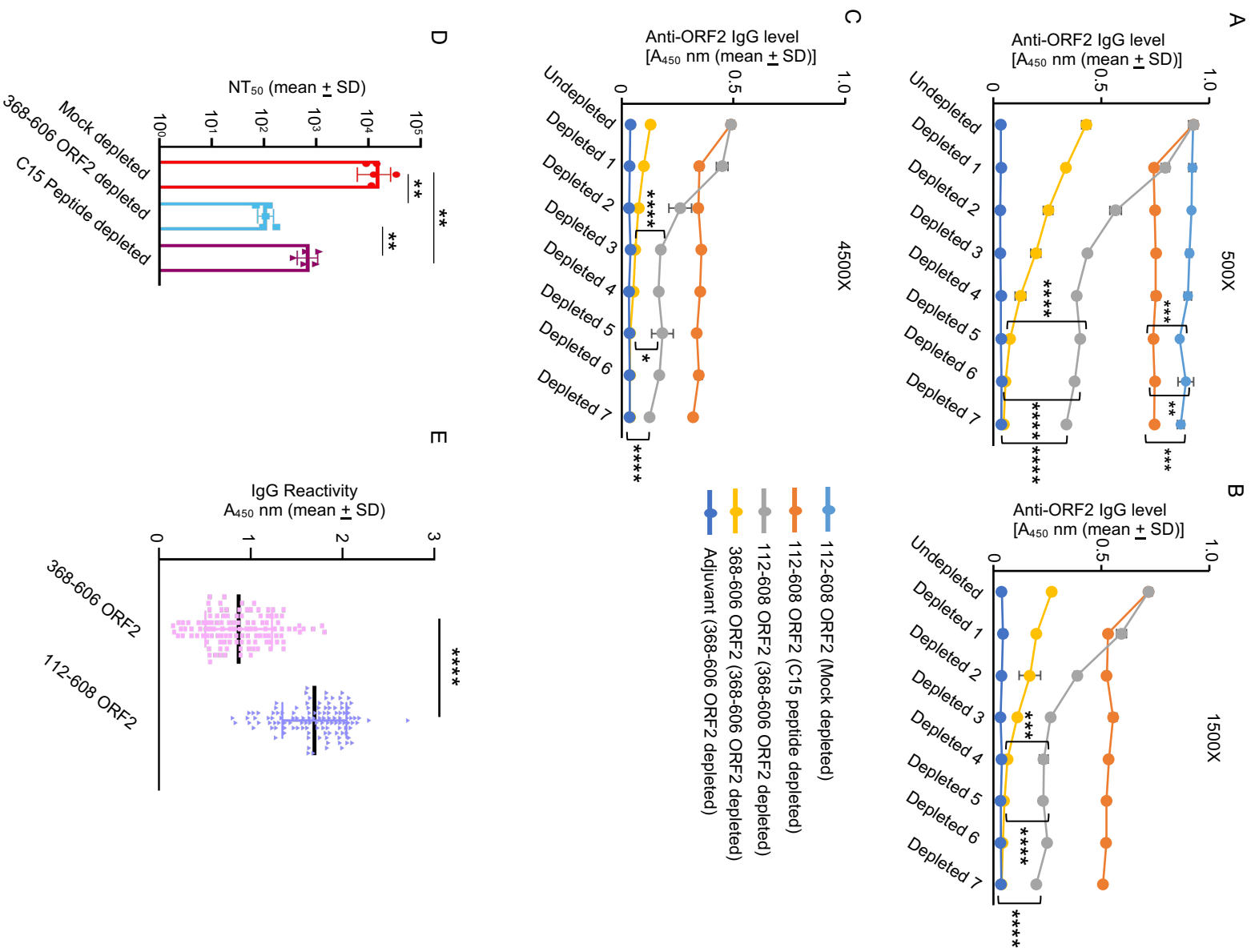


Figure 5: Immuno-depletion of 112-608 ORF2 protein immunized mice sera with 368-606 ORF2 protein or C15 peptide.

A) ELISA of immunodepleted 112-608 ORF2 immunized mice sera with 368-606 ORF2 protein or C15 peptide and immunodepletion of 368-606 ORF2 immunized mice sera with 368-606 ORF2 protein at 500X dilution. B) ELISA of immunodepleted 112-608 ORF2 immunized mice sera with 368-606 ORF2 protein or C15 peptide and immunodepletion of 368-606 ORF2 immunized mice sera with 368-606 ORF2 protein at 1500X dilution. C) ELISA of immunodepleted 112-608 ORF2 immunized mice sera with 368-606 ORF2 protein or C15 peptide and immunodepletion of 368-606 ORF2 immunized mice sera with 368-606 ORF2 protein at 4500X dilution. Serum collected on week 6 after first immunization were immunodepleted. ELISA was done in triplicate (n=3). D) NT_{50} of 112-608 ORF2 immunized mice sera after immunodepletion with 368-606 ORF2 protein or C15 peptide. Data is represented as mean $\pm$ SD and P-values were calculated using student t-test. ns: $P > 0.05$, (*): $P \leq 0.05$, (**): $P \leq 0.01$, (***): $P \leq 0.001$, (****): $P \leq 0.0001$. E) Scatter plot of 112-608 ORF2 and 368-606 ORF2 protein ELISA with HEV patient sera (n=147). A_{450} values of patient's sera was subtracted by average of the healthy sera values. P-value was calculated using student t-test. ns for $P > 0.05$, (*) for $P \leq 0.05$, (**) for $P \leq 0.01$, (***) $P \leq 0.001$, (****) $P \leq 0.0001$.

Supplementary Files

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