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1 Modelling of Hepatitis E virus RNA-dependent RNA polymerase genotype 3 from a chronic patient and *in silico*
2 interaction analysis by molecular docking with Ribavirin.

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4 Florencia Cancela^{1a}, Santiago Rendon-Marin^{2a}, Carolina Quintero-Gil², Douglas R. Houston³, Gediminas
5 Gumbis³, Yanina Panzera⁴, Ruben Pérez⁴, Juan Arbiza¹ and Santiago Mirazo^{1*}.

6
7 ¹ Sección Virología, Facultad de Ciencias, Universidad de la República. Iguá 4225. Montevideo, Uruguay. PC
8 11400. fcancela@fcien.edu.uy; jarbiza@fcien.edu.uy.

9 ² Grupo de Investigación en Ciencias Animales - GRICA, Facultad de Medicina Veterinaria y Zootecnia,
10 Universidad Cooperativa de Colombia, sede Bucaramanga. Carrera 33 B 30 A – 17. PC 680001.
11 santyrendon0519@gmail.com; dicquinterogi@unal.edu.co.

12 ³ Institute of Quantitative Biology, Biochemistry and Biotechnology, the University of Edinburgh.
13 DouglasR.Houston@ed.ac.uk; G.Gumbis@sms.ed.ac.uk.

14 ⁴ Sección Genética Evolutiva, Instituto de Biología, Facultad de Ciencias, Universidad de la República, Iguá
15 4225. Montevideo, Uruguay. PC 11400. ypanzera@fcien.edu.uy; rperez@fcien.edu.uy

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17
18 * Corresponding author: smirazo@fcien.edu.uy; Tel.: +598 2 5258618. Iguá 4225, Montevideo, Uruguay. PC
19 11400.

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21 ^a Both authors equally contributed to this work
22

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41 **Abstract**

42 Hepatitis E Virus (HEV) infection is an emergent zoonotic disease, where chronic hepatitis E associated to solid
43 organ transplant (SOT) recipients, related to genotype 3, is the clinical manifestation of major concern. In this
44 setting, ribavirin (RBV) treatment is the only available therapy, though drug-resistant variants could emerge
45 leading to a therapeutic failure. Crystallographic structures have not been reported for most of the HEV proteins,
46 including the RNA-polymerase (RdRp). Therefore, the mechanism of action of RBV against HEV and the
47 molecular interactions between this drug and RdRp are largely unknown.

48 In this work, we aimed to model *in silico* the 3D structure of a novel HEV3 RdRp (HEV_C1_Uy) from a
49 chronically HEV infected-SOT recipient treated with RBV and to perform a molecular docking simulation
50 between RBV triphosphate (RBVT), 7-methyl-guanosine-5'-triphosphate and the modelled protein.

51 The models were generated using I-TASSER server and validated with multiple bioinformatics tools. The
52 docking analysis were carried out with AutoDock Vina and LeDock software.

53 We obtained a suitable model for HEV_C1_Uy (C-Score=-1.33, RMSD=10.4 ± 4.6 Å). RBVT displayed a
54 binding affinity of -7.6 ± 0.2 Kcal/mol by molecular docking, mediated by 6 hydrogen-bonds (Q195-O14, S198-
55 O11, E257-O13, S260-O2, O3, S311-O11) between the finger's-palm-domains and a free binding energy of
56 31.26 ± 16.81 kcal/mol by molecular dynamics simulations.

57 We identified the possible HEV RdRp interacting region for incoming nucleotides or analogs and provide novel
58 insights that will contribute to better understand the molecular interactions of RBV and the enzyme and the
59 mechanism of action of this antiviral drug.

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70 1. Introduction

71 Hepatitis E virus (HEV) is a leading cause of acute hepatitis, a worldwide enterically-transmitted zoonotic disease
72 [1, 2] that cause large waterborne epidemic outbreaks in developing countries [3–6]. Nonetheless, in industrialized
73 countries, HEV has become an increasing public-health concern as autochthonous cases have raised drastically in
74 the last few years [7]. HEV is a small, 32-34 nm, non or quasi-enveloped particle with a single-stranded positive-
75 sense RNA genome of 7.2 Kb approximately [8]. The viral genome is capped at the 5'-end and polyadenylated at
76 the 3'-end, containing three partially overlapping open reading frames (ORFs) [9]. ORF1 encodes a non-structural
77 polyprotein of ~1693 amino acids (aa) with eight putative functional domains including methyltransferase,
78 protease, RNA-helicase and RNA-dependent RNA polymerase (RdRp) [10]. ORF2 encodes for the capsid protein
79 [11, 12] and ORF3 produces a multifunctional phosphoprotein essential for virion release [13, 14].

80 HEV is classified into the *Orthohepevirus A* genus of the *Hepeviridae* family and comprises eight distinct
81 genotypes (HEV1 to HEV8) [15]. HEV3 genotype is globally distributed [16] and is more prevalent in high-
82 income countries [17]. Outbreaks of this genotype are associated to the consumption of contaminated raw or
83 undercooked meat and liver sausages derived from pigs, the main reservoir of the disease [18, 19]. HEV3 infection
84 may evolve to a chronic hepatitis, particularly, in solid organ transplant (SOT) recipients and
85 immunocompromised individuals with lymphoma, leukemia and HIV infection [20–23], which could result in
86 liver fibrosis, cirrhosis or liver failure [24–27].

87 HEV belongs to the "alpha-like" supergroup III of positive-strand viruses, where HEV RdRp along with Rubella
88 virus RdRp (RUBV) and Beet necrotic yellow vein virus RdRp (BNYVV) conform a distinct close cluster [10].
89 In this supergroup, eight conserved motifs (I-VIII) have been described [10]. It has been suggested that HEV
90 RdRp can either initiate *de novo* synthesis from the RNA template or employ the template end to prime the
91 synthesis from the 3'OH end [28]. Localization studies revealed that HEV RdRp is present in the endoplasmic
92 reticulum (ER), suggesting the involvement of ER membrane in HEV replication [29].

93 Even though HEV RdRp is a crucial viral protein, information about its functional domains, template specificities,
94 nucleotide selection, structure and functional role have not been described yet.

95 Ribavirin (RBV) (1-b-D-ribofuranosyl-1,2,4-triazole) is a guanosine/adenosine synthetic analog with broad-
96 antiviral spectrum [30] and is phosphorylated intracellularly by a cellular adenosine kinase to monophosphate
97 (RBVM), diphosphate (RBVD) and triphosphate (RBVT) forms [31]. RBVM is a competitive inhibitor of inosine
98 monophosphate dehydrogenase that leads to a depletion in the GTP pools needed for RNA viral synthesis [31].
99 Moreover, RBVT can be misincorporated by the RdRp in the nascent viral RNA inducing base transitions,

100 producing an early chain termination and inhibition of replication by competitively inhibiting the binding of
101 nucleotides [31]. Modeling studies revealed that the base moiety of ribavirin, 1,2,4-triazole-3-carboxamide, forms
102 base pairs with both cytosine and uracil by a rotation of the carboxamide group [32]. Furthermore, it was observed
103 that once RBV is in the RNA, it could be trapped in the "anti" conformation of the "pseudo-base" and is then able
104 to bind base pairs with incoming pyrimidines [32]. For these reasons, RBV has been also considered a viral
105 mutagen that could exceed the "error-catastrophe" threshold in RNA viruses, causing mainly A to G and U to A
106 substitutions [33] that leads to lethal mutagenesis [31, 32, 34–37].

107 Until several years ago, RBV was employed to treat Lassa fever virus infection [38–40] and in combination with
108 interferon- α to treat hepatitis C virus (HCV) infection [41–43], vesicular stomatitis virus [44, 45], La Crosse
109 encephalitis virus [46], reovirus [47], influenza [48, 49] and human immunodeficiency virus [50].

110 Currently, RBV is used as a monotherapy only to treat respiratory syncytial virus infection under certain clinical
111 circumstances [51–53].

112 In the case of chronic HEV infection, in which viral RNA remains detectable for at least 3-6 months, there is no
113 specific antiviral treatment for the patients. The first therapeutic line consists in decreasing the
114 immunosuppression therapy, which exhibits good outcome in only 30% of the patients [25]. In a high proportion
115 of cases where HEV clearance could not be achieved, RBV is administered for another 3 months [54], with good
116 results [24, 55–58]. However, RBV treatment failure has been reported frequently due to HEV antiviral resistance
117 likely associated to G1634R, Y1320H and K1383N substitutions in the RdRp [59]. The K1383N substitution is
118 located in the F1-motif (177-180 aa in HEV RdRp) which is believed to bind the incoming nucleotide-triphosphate
119 and select the correct one [60]. Hence, RBV has been proposed to be mutagenic to HEV during a prolonged
120 treatment. Interestingly, the K1383N substitution could strongly decrease viral replication and increase RBV
121 sensitivity *in vitro*, opposite to the observed clinical phenotype [59]. On the other hand, the Y1320H substitution
122 increases HEV replication without altering RBV sensitivity, which may then be a compensatory change for the
123 fitness loss resulting from K1383N. The G1634R substitution seemed to increase the replicative capacity of HEV
124 and then reduce the efficiency of RBV [61].

125 In this work we pursued two goals. First, to obtain an HEV3 RdRp 3D *in silico* model of a strain coming from a
126 chronic HEV-SOT patient treated with RBV [62], as there is no crystallographic structures available in databases,
127 in order to thoroughly analyze HEV RdRp structural characteristics. Second, to perform a comprehensive
128 molecular docking simulation between RBVT and the modelled HEV protein, aimed to provide novel insights on
129 the enzyme-antiviral drug molecular interaction.

130 **2. Materials and Methods**

131 **2.1. HEV strain and RNA extraction.**

132 The HEV strain employed in this study came from a chronic hepatitis E case belonging to genotype 3 recently
133 reported in Uruguay by our group (HEV_C1_Uy) [62]. The patient was a liver-transplanted (LT) 62-year-old man
134 presenting an autochthonous chronic HEV3 infection with an altered liver enzymogram and histologic evidence
135 of HEV infection (1 year and 6 months after LT), who was then treated with a 9-weeks course of RBV (1200
136 mg/day). After the RBV course he had a sustained virological response (SVR) in the 24 months follow-up.

137 Total RNA was extracted from a 10% fecal PBS suspension with *Quick-RNA*[™] Miniprep Kit (Zymo Research
138 Corp, USA) following manufacturer's instructions.

139 The consensus nucleotide sequence of the HEV RNA polymerase here described was submitted to GenBank under
140 the accession number MT774175.

141

142 **2.2. Next Generation Sequencing (NGS) and data analysis**

143 Double stranded cDNA (dscDNA) was generated using Maxima H Minus Double-Stranded cDNA Synthesis Kit
144 (ThermoFisher Scientific, USA) with random primers and 12 µL of extracted RNA. The dscDNA was amplified
145 by Multiple Displacement Amplification (MDA) technology using REPLI-g Mini Kit (Qiagen, Germany)
146 followed by purification and quantification using AMPure XP (Beckman Coulter, USA) and a Qubit fluorometer
147 (Qubit[™] DNA-HS Assay kit), respectively.

148 Nextera DNA Flex Library Preparation kit (Illumina, USA) with dual indexing was used from 50 ng of dscDNA.

149 Control quality libraries were performed on a Fragment Analyzer 5200 system (Agilent Technologies, USA) using
150 the Standard Sensitivity NGS Analysis Kit (Agilent Technologies, USA). Library was sequenced on an Illumina
151 MiniSeq Genomic Platform at the Faculty of Sciences (UdelaR, Uruguay) using Mid Output Reagent Cartridge
152 (300-cycles, 150 base-pair paired-end reads) by following standard Illumina protocols.

153 Sequencing raw reads were demultiplexed automatically on the MiniSeq platform with the default settings.

154 Adapter/quality trimming and filtering were performed with BBDuk plugin and clean reads were mapped to a
155 hepatitis E genome (FJ998008) using Geneious mapper (medium-low sensitivity) available in the Geneious Prime
156 2020.2.1 software (<https://www.geneious.com>).

157 Reference assembly and annotation was done with SeqMan NGen[®] Version 12.0 (DNASTAR, Madison, WI)
158 using the reference genome retrieved from the GenBank database FJ998008.

159

160 **2.3. RdRp sequence alignments**

161 The RdRp domain was identified from the HEV_C1_Uy complete genome according to other HEV polymerase
162 annotations in Uniprot database [63].

163 Multiple RdRp nucleotide and amino acids (aa) sequence alignments of complete RdRp from HEV strains and of
164 the catalytic site from HEV and other viruses were carried out with ClustalW in MEGA v.7. [64]. HEV_C1_Uy
165 complete RdRp and HEV3 selected subtype reference strains 1a-8a [65] were included for the alignment (Table
166 S1).

167 An alignment including only the catalytic site of different viral RdRp was also carried out for HEV_C1_Uy and
168 HEV reference strains (Table S1).

169 Additionally, diverse RdRp sequences were selected from several viruses for further analysis: Hepatitis C virus
170 1a-*Flaviviridae*, Hepatitis A virus IB- *Picornaviridae*, Coxsackievirus B1-*Picornaviridae*, Norwalk virus of the
171 *Caliciviridae* family (former HEV classification), RUBV-*Matonaviridae* (reported to exhibit conserved motifs
172 with HEV RdRp [10]) and BNYVV-*Benyviridae* a plant furovirus belonging to the "alpha-like" supergroup. Top
173 first threading templates and structural analogs from I-TASSER modelling were also added to the alignment from
174 RCSB:PDB database [66] (Table S1). Viral RdRp catalytic site annotated sequences were obtained from Uniprot
175 database. P-distance pairwise matrices for RdRp catalytic site between HEV strains and the other viral sequences
176 were performed in MEGA v. 7. A phylogenetic tree for the RdRp catalytic sites was constructed in MEGA v.7 by
177 the Neighbor-Joining method with the Poisson model as the best substitution model. Bootstrap values were
178 determined with 1000 replicates of the dataset.

179

180 **2.4. HEV RdRp 3D modelling, structural analysis and validation**

181 The amino acid sequences from the HEV_C1_Uy RdRp and a HEV3 RdRp from the swine Arkell strain (reference
182 of subtype 3j) were employed to determine the 3D-structure. The secondary structure was predicted using
183 PSIPRED 4.0 tool [67] (UCL-CS Bioinformatics). Annotation information was retrieved from ExPASy-PROSITE
184 [68] and Pfam database [69]. Chemical and physical parameters for this protein were obtained from ExPASy-
185 ProtParam tool [70].

186 Thorough sequence alignments analyses were performed in order to evaluate HEV RdRp homology modelling
187 possibility, which was then discarded (data not shown). Therefore, the I-TASSER prediction server [71] was
188 employed to obtain the 3D structure models and the best ranked structure in the hierarchical analysis in terms of
189 the best C-Score and Root Mean Square Deviation (RMSD) were selected [71].

190 The models were assessed and validated using bioinformatics tools. ProSA-Web [72] was used to calculate the Z-
191 score for the overall model quality which enables to establish whether the Z-score value of the model structure is
192 located in the range of Z-scores exhibited by native proteins of similar size, with PDB as reference database. The
193 Ramachandran plots (RAMPAGE) [73], were also constructed to establish amino acids in energetically favorable
194 regions. Additionally, ERRAT [74], Verify 3D [75] and PROCHECK [76] software were employed. All these
195 computational tools enable us to determine whether 3D models of HEV RdRp are reliable models to employ in
196 molecular docking analysis.

197 The generated models were structurally aligned to the best ranked structures to establish the RMSD differences
198 between the model and best template structures utilizing TM-Align based on TM-score [77]. The crystallized
199 structures (6R1I, 1SH0, 3N6L, 3CDU) were downloaded from the PDB database.

200 Molecular graphics were performed with UCSF Chimera v. 1.8. [78].

201

202 **2.5. Molecular docking**

203 The 3D structure of RBV and RBVT were downloaded in PDB format from PubChem-NIH [79] (CID 37542 and
204 122108, respectively). 7-methyl-guanosine-5'-triphosphate (GTP) and Cinnamaldehyde (CIN) 3D structures were
205 downloaded in PDB format from the Drugbank database [80] (Accession Numbers DB02716 and DB14184,
206 respectively). The GTP physiological nucleotide was employed as an internal control for the molecular interaction
207 analyses. The CIN organic compound, which is the main component of the cinnamon, was included as a negative
208 control since no affinity towards RdRp has been reported for this compound [81].

209 Interaction analysis of HEV_C1_Uy RdRp and HEV Arkell RdRp with RBV, RBVT, GTP and CIN were carried
210 out with AutoDock Vina v. 1.1.2 [82]. The grid box was defined with the online tool PeptiMap [83], which predicts
211 the best ligand-binding site on the protein surface. The models were minimized adding charges with the default
212 parameters in PMV v.1.5.6 (MGLTools-The Scripps Research Institute) [84] and saved in PDBQT file formats
213 for the docking. Five runs of each docking assay were performed in a grid box with a spacing of 1 Å, presenting
214 the dimensions $x=24$, $y=28$, $z=22$ and its center located in $x=74.853$, $y=63.244$, $z=55.892$ for HEV_C1_Uy
215 RdRp. Concerning the HEV Arkell RdRp, conditions were the same as aforementioned but with dimensions $x=$
216 24 , $y=24$, $z=28$ and the following coordinates $x=65.309$, $y=56.220$, $z=61.801$.
217 Additional docking assay was carried out involving the analog sites between HCV-ribonucleoside triphosphates
218 (rNTPS) interaction and HEV_C1_Uy RdRp and HEV Arkell RdRp. The grid box for HEV_C1_Uy RdRp had
219 the following characteristics, size: $x=20$, $y=24$, $z=28$, center: $x=80.208$, $y=63.928$, $z=60.218$, whereas for HEV

220 Arkell RdRp presented size: $x=30, y=24, z=20$; center: $x=64.757, y=52.581, z=68.09$. All the docking studies
221 were also run five times in LeDock [85], employing the same grid box coordinates as in AutoDock Vina, with the
222 ligand file format in SYBYL Mol2. In all cases, binding energies were reported as the mean Kcal/mol \pm SD.
223 The 2D protein-ligand interaction diagrams were built employing the software LigPlot+ v. 4.5.3 [86] and
224 PoseView-ProteinsPlus [87]. Analysis of the generated 3D docking interactions were performed in UCSF
225 Chimera v. 1.8.

226

227 **2.6. Molecular dynamics simulation and binding free energy calculations.**

228 mol2 files for Cinnamaldehyde (ZINC1532777), RBVT (ZINC12402860), and 7-methyl-GTP (ZINC15601432)
229 were downloaded from ZINC Database [88]. These files were used for parametrization of the ligands with the
230 help of SwissParam [89]. Using VMD, HEV_C1_Uy RdRp-ligand complexes were made, solvated, and,
231 subsequently, the solvation box was ionized with 0.15 M of Na⁺ and Cl⁻ [90]. Molecular Dynamics (MD)
232 simulations followed; for this, we utilized NAMD 2.14 [91]. Minimization was carried out for 10,000 steps while
233 a step itself was 2 fs long. Following minimization, the three systems were equilibrated for 2 ns, and, finally,
234 longer MD simulations were carried out so that the total length of the simulations were at least 100 ns. CaFE [92]
235 was used to perform molecular mechanics Poisson-Boltzmann surface area (MM/PBSA) calculations. CaFE
236 utilized NAMD for the MM component, APBS for PB calculations [93], and VMD for SA calculations.
237 Trajectories derived from the MD simulations were analyzed using CaFE. Default parameters of CaFE were used
238 for the calculations.

239

240 **3. Results**

241 **3.1. HEV_C1_UY RdRp showed non-reported amino acidic substitutions**

242 The HEV3 C1 Uy complete genome from the HEV-SOT chronic patient was successfully obtained by Illumina
243 sequencing with a mean average coverage of sequencing depth of 71.7.

244 The RdRp domain was identified at the 1217-1703 position of the nonstructural protein. This protein contained
245 487 residues, a molecular weight of 53526.49 Da and an isoelectric point of 5.94.
246 Due to the high sequence divergence of the complete RdRp sequence among viral families, the alignments and
247 identity matrices analyses were conducted separately for the catalytic site. Sequence analysis of the catalytic site
248 revealed an identity for HEV_C1_Uy RdRp ranging from 17% for Coxsackievirus B1 and B3 to 29.8%-26.6%
249 for BNYVV and RUBV, respectively. The percentage of identity of HEV_C1_Uy with HEV3 reference strains

250 ranges from 95.7% (3e) to 98.9% (3a, 3i and 3k). HEV_C1_Uy RdRp showed 86.89% of sequence identity with
251 the closest related isolate (FJ705359).

252 HEV_C1_Uy presented several non-reported and infrequent substitutions, which were located outside the
253 catalytic site. At the amino acidic level, a unique non-reported substitution V/I4A (V/I1220A referred to ORF1
254 position) was found in HEV_C1_Uy strain. The changes T/A392V (T/A1608V) and V443A (V1659A) in
255 HEV_C1_Uy were exclusively found in HEV8a and 5a genotypes, respectively, which are very distantly related
256 to HEV3, whereas the HEV_C1_Uy Q484H (Q1700H) change was previously reported in few strains from 2a, 5a
257 and 6a genotypes (Fig. 1).

258 The eight reported conserved motifs (I-VIII) of the positive-strand viral RNA RdRp [10] were identified among
259 the sequence alignments (Table 1). Motifs IV (D-x(4,5)-D) and VI (GDD) in the catalytic site were conserved
260 among HEV strains and other viral families (Fig. 1).

261 HEV proteins have not clustered with any other RdRp viral sequence but were closely related and shared the
262 highest homology with the RUBV and BNYVV proteins (Fig. 2). Analysis at the nucleotide level, exhibited that
263 HEV_C1_Uy formed a separate cluster from other HEV3 subtypes strains (data not shown).

264

265 **3.2. An acceptable HEV_C1_Uy RdRp 3D structure was obtained**

266 HEV_C1_Uy RdRp and HEV Arkell RdRp models obtained had a C-Score of -1.33, RMSD score of 10.4 ± 4.6
267 Å and a C-Score of -1.10, respectively. C-score values determines the model quality and ranges from [-5, 2],
268 where a higher value means a higher confidence. Additionally, a C-Score > -1.5 supports a model with correct
269 global topology. The RMSD values determines the divergence in angstroms between the modelled protein and its
270 template. Therefore, the obtained HEV RdRp models exhibited a suitable global topology.

271 The top first templates used by I-TASSER to model the proteins corresponded to the Porcine Aichi virus
272 (Kobuvirus) from the *Picornaviridae* family (6R1I), Norwalk virus (1SH0) and Enterovirus A71 (EV71)-
273 *Picornaviridae* (3N6L). The top first structural analog obtained with I-TASSER for HEV_C1_Uy RdRp
274 corresponded to Coxsackievirus B3 (3CDU).

275 These models were further validated employing different bioinformatics tools. PROCHECK server, which
276 verified the stereochemical quality of a protein structure, analyzing each residue geometry and the general
277 geometry, showed the best results. Similarly, acceptable Ramachandran plots were observed between the models
278 (86.60%-HEV_C1_Uy and 84.10%-Arkell corresponding to aa in favorable region for torsions and rotations).

279 Moreover, the crystallographic structures used as templates were also validated for comparison. Overall, the two
280 models exhibited acceptable validation values (Table 2).

281 Additionally, structural alignments between the two models, and between the two models and the templates were
282 carried out. TM-Score for HEV_C1_Uy and HEV Arkell was 0.86, similar values were observed for the PDB
283 templates. The highest TM-Scores registered were 0.88 for HEV_C1_Uy and the Enterovirus A71 and of 0.91 for
284 HEV Arkell and Porcine Aichi virus. TM-Score are in the range of (0, 1], where 1 corresponds to a perfect match
285 between two structures and values greater than 0.5 indicates the same fold. Therefore, all the TM-Scores obtained
286 suggested that the structure of the models and the templates had the same fold. These results reinforce the
287 confidence in the obtained structural models.

288 The secondary structure prediction chart for the HEV_C1_Uy model exhibited the distribution of the helix, strand
289 and coil throughout the protein sequence and the confidence of prediction for each section (Fig. 3).

290 The finger's, thumb and palm domains were identified in the 3D models (Fig. 4). Also, the 3D structural alignment
291 revealed that HEV_C1_Uy and HEV Arkell, as well as HEV_C1_Uy and EV71 had similar structural folding as
292 observed by the TM-align Scores (Fig. 4).

293

294 **3.3. HEV_C1_Uy exhibited very favorable binding affinity with RBVT.**

295 Molecular docking studies between RBVT and the modelled RdRp were executed in order to evaluate their
296 interaction interface. HEV_C1_Uy RdRp and the control protein HEV Arkell showed very favorable binding
297 energies with RBVT in AutoDock Vina (-7.6 ± 0.2 Kcal/mol and -8.0 ± 0.1 Kcal/mol, respectively) and LeDock
298 (-8.01 ± 0.18 Kcal/mol and -8.16 ± 0.14 Kcal/mol, respectively) (Table 3). In fact, these values were under the -
299 7.0 Kcal/mol threshold [94], and are indicative of a strongly binding ligand; this threshold was defined for a set
300 of diverse antivirals ligands in Auto Dock against Human Immunodeficiency Virus (HIV). The interactions were
301 mediated by 6 hydrogen bonds (H-bond) in the case of HEV_C1_Uy involving residues Q195-O14, S198-O11,
302 E257-O13, S260-O2, O3, S311-O11 and 9 H-bonds for HEV Arkell. The closest distance between atoms of RBVT
303 and HEV_C1_Uy was of 2.88 Å and the more distant one was of 3.07 Å (Fig. 5).

304 The GTP interaction used as control showed the best binding affinity for HEV_C1_Uy. On the other hand, in
305 HEV Arkell the most favorable interaction for AutoDock Vina corresponded to RBVT. However, according to
306 LeDock the best score obtained was for GTP. Furthermore, the negative control (CIN) exhibited a weak binding
307 energy (-5.0 ± 0 Kcal/mol and -2.99 ± 0.03 Kcal/mol with AutoDock Vina and LeDock, respectively), indicating
308 a non-specific interaction. Unphosphorylated RBV was also evaluated by molecular docking as control, (data not

309 shown), though further 3D interaction analyses were carried out with RBVT, since it is the active form for RdRp
310 interaction.

311 Additionally, since the residues for HCV and rNTP H-bond interaction have been previously identified [95],
312 through sequence alignment we extrapolated them to HEV_C1_Uy RdRp to carry out RBVT docking studies in
313 those sites for comparison purposes. The analog sites HEV-HCV were: S367-Q262, R386-R291, R394-G305 and
314 T390-E301. Highly similar favorable binding affinity was observed (-7.7 ± 0.2 Kcal/mol for RBVT with
315 AutoDock Vina) compared to the previously mentioned molecular docking with the Peptimap prediction sites ($-$
316 7.6 ± 0.2 Kcal/mol). The interaction of RBVT-HEV_C1_Uy in this case was mediated by 7 H-bonds, I197-N4,
317 S198-O14, N4, S260-O4, K309-O12, O13, S311-O13, with 2.82 Å and 3.08 Å as the closest and furthest distance
318 between atoms, respectively. Several interacting amino acids are the same that those observed with Peptimap
319 sites.

320

321 **3.4. Molecular dynamics simulation analysis.**

322 To a large degree, MM/PBSA results are in agreement with the docking results. For CIN, the estimated free
323 binding energy to HEV_C1_Uy RdRp is in the expected range (-7.65 ± 4.16 kcal/mol) when standard deviation
324 (SD) values are taken into account. For GTP, when taken together with a considerable SD value, the free binding
325 energy estimate confirms expectations (5.38 ± 12.69 kcal/mol). As for RBVT, the MM/PBSA results predict a
326 binding affinity of 31.26 ± 16.81 kcal/mol to HEV_C1_Uy RdRp.

327 All simulations reached equilibrium states (Fig. 6 A-C). Little fluctuation was seen in the residues, except for the
328 range 50-200 (which contains the residues that form the binding pocket and some flexible loops) and, as would
329 be expected, the termini (Fig. 6 D-F).

330

331 **4. Discussion**

332 Chronic hepatitis E in SOT recipients and immunocompromised individuals, frequently associated to severe
333 extrahepatic manifestations, is a disease of major concern in high-income and non-endemic countries where HEV3
334 is prevalent. In the last few years, product of a sharp increment of HEV cases and an improvement of the diagnosis,
335 many aspects of chronic HEV infection have been uncovered and much more information is now available [96].
336 In this clinical setting, antiviral treatment with RBV, the only approved drug to treat HEV infection, is the main
337 option since the risk of acute rejection prevents the use of pegylated interferon alpha as a therapeutic option [55,

338 57, 58]. In fact, RBV administered for at least 3 months has shown favorable outcomes with confirmed efficacy
339 in acute and chronic HEV cases [24, 55–58].

340 Unfortunately, though significant advances have been achieved in terms of cell-culture isolation of HEV [97, 98]
341 the lack of an efficient and standardized model has hampered the study of HEV viral cycle and therefore prevented
342 the comprehension of the antiviral mechanisms of action, of RBV. Viral RdRp is believed to interact with RBV,
343 though the molecular details of HEV-RBV interaction have not been identified yet. Remarkably, no HEV RdRp
344 enzyme have been crystallized and the likely ligand-binding pocket for nucleotides or any other molecule is not
345 known. This contrast with other viral RdRp which have been co-crystallized with nucleoside triphosphates or
346 oligonucleotides to map the substrate-binding sites, as reported for HCV-rNTPs complex [95]. Hence, *in silico*
347 computational approaches, which are widely employed to predict and evaluate molecule-target interactions for
348 drug discovery [99, 100], might be an useful tool for afford this knowledge gap. Structural studies on replicative
349 complexes of RdRp and NTPs or analogs are currently needed to better understand the enzymes low copying
350 fidelity and the mutagenic activity of the analogs on the viral replication [101].

351 In this work, we pursued two goals. First, to model *in silico* the HEV3 RdRp protein of a HEV chronic strain and
352 second, to perform a detailed molecular docking study and molecular dynamics simulations, with the aim to
353 identify the interacting domain of the viral enzyme and its binding affinity with the drug RBV.

354 Herein, we report a complete HEV RdRp sequence (HEV_C1_Uy), corresponding to a LT patient chronically
355 infected with HEV3. The patient had been successfully treated with RBV in a 9-weeks course, and had an SVR
356 during the 24 months follow-up. Sequence analysis identified the HEV_C1_Uy catalytic site corresponding to the
357 region spanning residues 248 to 359. This catalytic site showed elevated sequence divergence among virus
358 families, being the highest aa percentage identity observed with BNYVV and RUBV (29.8%-26.6%,
359 respectively). These results support the notion that these viruses constitute a distinct monophyletic group in the
360 "alpha-like" supergroup of positive-strand RNA viruses [10]. However, HEV3 strains formed a separate
361 independent cluster from the RUBV and BNYVV group in the phylogenetic group reconstruction.

362 RNA and DNA polymerases share a basic structure, where RdRp are more similar to each other than to other
363 different polymerases. There is almost no detectable sequence similarity between viral RdRps with the exception
364 of some conserved motifs [102, 103]. Indeed, this was the case with HEV and other virus, since sequence
365 alignments needed to be performed including only the catalytic site due to the extreme divergence observed.
366 Interestingly, all these diverse viruses have two conserved motifs, D-x(4,5)-D and GDD. It has been shown, that
367 *in vitro* substitutions in the GDD motif abolished the RdRp activity of HEV [104], HCV [105], RUBV [106],

368 calicivirus [107] and poliovirus [108]. Thus, the GDD motif plays a crucial role in the catalytic activity and metal
369 ion coordination [109, 110], which could therefore explain its conservation among a wide range of RdRps.

370 On the other hand, there is enough sequence conservation in order to perform alignments and identify motifs
371 within some viral families [102]. Certainly, this was observed for HEV and RUBV alignment, where eight
372 conserved motifs (I-VIII) associated to positive-strand viral RNA RdRps were successfully identified.

373 Furthermore, sequence comparison between HEV_C1_Uy with HEV genotypes and HEV3 subtypes revealed
374 several differences, where few unique (V/I1220A) and infrequent substitutions (T/A1608V, V1659A and
375 Q1700H) were observed. Single nucleotide variants at protein level have been reported to be less abundant at
376 ligand binding sites and less rare variants were found to be located apart from enzyme active sites, resulting in
377 moderate changes of the physic-chemical properties of the aa [111]. Moreover, it has been reported that mutations
378 in the functional sites would alter the enzyme's catalytic activity, even mutations with no significant effects can
379 modify the affinity of protein-drug interactions [112]. Current *in vitro* research is being conducted to address if
380 these HEV_C1_Uy substitutions, which were distantly located from the catalytic site, are involved in RBV
381 sensitivity or in the enzymatic activity. Debing et al. [59] reported three substitutions likely associated to RBV
382 resistance (Y1320H, K1383N and G1634R) located outside the catalytic site. Additionally, other substitutions
383 have been reported in HEV infected patients (D1384G, K1398R, V1479I, Y1587F), which were suggested to be
384 replication competent and to possibly affect the HEV replication by modulating the RdRp activity [61, 113–115].
385 Recently, substitutions mutants C1483W and N1530T isolated from HEV acute liver failure patients have been
386 strongly associated to high viral load and mortality [116].

387 Notably, a RdRp substitution was reported (F1439Y) to be significantly associated to HEV fulminant liver failure
388 patients [117]. Among the reported substitutions, HEV_C1_Uy RdRp presented F1439Y (F233Y in HEV_C1_Uy
389 RdRp) and V1479I (V273I). The V1479I substitution has been previously reported in chronic HEV-SOT patients
390 exhibiting RBV resistance, suggesting that this substitution could modulate the RdRp activity [115].

391 Interestingly, the HEV3 Arkell swine strain selected for modelling and docking comparison, presented the
392 G1634R and the F1439Y substitutions.

393 Moreover, RBV has been suggested to act as a mutagen in patients chronically infected with HCV [118]. Several
394 studies showed that *in vitro* growing of poliovirus (*Picornaviridae*) in the presence of RBVT promotes the
395 selection of the mutant G64S, that showed a lower affinity to RBVT, thus increasing template copying fidelity
396 [119–122]. Furthermore, it has been demonstrated that the G64R, G64T and S264L substitutions confer RBV
397 resistance in EV71 by increasing the RdRp replication fidelity [123]. Therefore, RBV may indeed exert its

398 antiviral activity through a mutagenic effect also for HEV [124–126], as it has been reported that RBV increases
399 HEV quasispecies heterogeneity [59, 61, 127, 128].

400 Validated HEV3 C1 Uy RdRp and HEV Arkell models were obtained through bioinformatics *de novo* strategies.
401 These models exhibited a correct global topology and share the same folding with a high structural alignment
402 score (0.91).

403 Previous reports have demonstrated that it is possible to acquire a reliable 3D RdRp model *in silico*, even when
404 there is low sequence identity with modelling templates [129]. The widely employed I-TASSER server is a
405 powerful platform based on sequence-to-structure-to-function prediction paradigm, where the software first
406 generates three-dimensional atomic models from multiple threading alignments and iterative structural assembly
407 simulation. The threading methodology is used for identifying template proteins from solved structure databases
408 that have a similar structure or similar structural motifs [130].

409 RdRps share a similar overall structure with the finger's, thumb and palm domain arranged in a cupped right-hand
410 configuration, with an N-terminal domain bridging the finger's and thumb region [131], which were successfully
411 identified in the HEV models.

412 Molecular docking analysis for RBVT with HEV_C1_Uy and HEV Arkell revealed a favorable binding affinity
413 under the established threshold (-7.0 Kcal/mol) [94], with similar good values observed for the GTP control. The
414 RBVT interactions were mediated by 6 H-bond for HEV_C1_Uy (Q195-O14, S198-O11, E257-O13, S260-O2,
415 O3, S311-O11) and 9 H-bonds for HEV Arkell (H6-O7, M67-O12, Q69-O12, E70-O12, K177-O3, Q195-O3,
416 G196-O10, S198-O7, K309-O9). These interaction sites were very similar between both models since similar
417 regions were identified as best candidates for docking by Peptimap and were found to be buried between the
418 finger's-palm domains of the HEV RdRp. Notably, the GTP binding region was located within the same
419 interacting site for RBVT in the HEV models, suggesting that this is the region that could directly interact with
420 incoming nucleotides or analogs.

421 The RdRp structure of poliovirus (*Picornaviridae*) has a N-terminal glycine residue buried in a pocket at the base
422 of the finger's domain, forming 4 H-bonds that reposition the catalytic residue Asp238 into the active site (palm
423 domain). The Asp238 residue was then able to establish a long H-bond interaction (2.8 Å) with the 2'OH of the
424 incoming rNTP, as part of a flexible interdomain linker, a common molecular mechanism to most picornaviruses
425 [132, 133]. A similar atom distance was obtained for RBVT-HEV_C1_Uy (2.82-3.07 Å). Indeed, HEV_C1_Uy
426 and EV71 (*Picornaviridae*) exhibited the best structural alignment score, as well as HEV Arkell and Porcine Aichi
427 virus, another *Picornaviridae* family member.

428 Substitutions V/I1220A, T/A1608V, V1659A and Q1700H identified in HEV_C1_Uy, occur in the finger's,
429 thumb, thumb and in the protruding coil-thumb domain, respectively, while the substitutions F233Y and V273I
430 were located in the bridge thumb-palm domain and in the finger's domain, respectively. These positions and
431 particularly the domains where they are located were described in several viral families. To gain insight into the
432 mutational effect of RBV, the X-ray structure of the foot-and-mouth disease virus (FMDV) (*Picornaviridae*) with
433 natural substrates (ATP, UTP) and RBV had been obtained and reported [101]. It was shown that the loop β 9-
434 α 11 of the finger's domain, can be flexible and necessary to adapt its conformation and interactions to the size
435 and shape of the incoming nucleotides and, additionally, it contains the M296I substitution found in RBV resistant
436 FMDV strains [101]. Conversely, the HCV 3D RdRp modelling revealed that the F415Y RBV resistant variant is
437 located at the P helix region of the thumb domain, which is suggested to interact with the minor groove of the
438 template-primer duplex in the putative-RNA binding site [33]. Furthermore, HCV-rNTPs reported binding sites
439 [95] were close to the RBV interaction region in our HEV models, with favorable docking simulation scores.

440 To explore into the stability of the HEV_C1_Uy-ligand complexes, further analysis was performed by a 100 ns
441 MD simulation. The binding free energy calculations by MM/PBSA confirmed the previous docking results
442 obtained for GTP and CIN taking the SD values into consideration. However, the MM/PBSA results for RBVT
443 did not correspond to the favorable binding affinity observed by molecular docking. This observation is very
444 interesting and raises additional questions concerning the role of selected mutations in the RBV antiviral activity.

445 One explanation of this result might be that the HEV_C1_Uy strain presents several unique and infrequent
446 substitutions in the RdRp protein (V/I1220A, T/A1608V, V1659A and Q1700H), as well as one associated with
447 fulminant liver failure (F1439Y) and one identified in RBV resistant patients (V1479I), suggested to be involved
448 in RdRp activity modulation. Therefore, this rare combination of substitutions might affect the binding stability
449 of RBVT-HEV_C1_Uy complex. Particularly, the presence of the substitution V/I1220A, not previously reported
450 in an HEV-RdRp, was located within the RBVT binding-pocket analyzed, which could then alter this complex
451 binding affinity. Further reverse genetics-based *in vitro* research will be needed to shed light on this issue.

452 On the other hand, the MD force field may not represent the highly polar phosphate groups well, as for instance,
453 it takes no account of the possibility of different ionization states or dynamic polarization effects induced by the
454 interacting protein groups. MD simulations are also heavily dependent on the initial ligand conformation, so even
455 a slight difference in ligand conformation would affect the binding affinity values [134, 135].

456 In summary, by using a bioinformatics approach, we obtained the first acceptable models of HEV RdRp belonging
457 to a viral strain isolated from a chronically infected patient and the reference HEV3 Arkell swine strain, in order
458 to perform molecular docking studies and MD simulations with RBVT.

459 Results described here showed that RBVT could bind to the HEV3 RdRp finger's-palm domains, and the possible
460 interaction site and H-bonds involved are described in detail. We also showed that RBVT and GTP might share
461 the same binding site in the RdRp, suggesting that this could be the interacting region for incoming nucleotides
462 or analogs. However, MM/PBSA results differed from the binding affinities obtained by molecular docking for
463 the reasons previously mentioned.

464 Even tough additional research efforts should be performed *in vitro* aimed to corroborate all these data, our
465 findings will contribute to better understand the mechanism of action of RBV in HEV RdRp, and therefore, this
466 validated model could be an useful tool for the development of new potential HEV antiviral drugs on a rational
467 basis by inferring the possible ligand-target interaction.

468

469 **Author Contributions**

470 Conceptualization, C.Q-G and F.C.; methodology, F.C., S.R-M., Y.P., R.P., D.R.H; software, F.C., S.R-M.
471 D.R.H., G.G.; validation, F.C. and S.R-M.; formal analysis, F.C., S.R-M., C.Q-G. D.R.H., G.G and S.M.,;
472 investigation, F.C.; S.M.; resources, S.M.; data curation, Y.P., R.P.; writing—original draft preparation, F.C.;
473 writing—review and editing, C.Q-G., S.R-M., Y.P., R.P., D.R.H., G.G. and S.M.; visualization, F.C., S.M.;
474 supervision, C.Q-G. and S.M.; project administration, S.M, J.A.; funding acquisition, S.M. and J.A. All authors
475 have read and agreed to the published version of the manuscript.

476

477 **Disclosure statement**

478 The authors declare that there are no conflicts of interest.

479

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485

486 **Ethical approval**

487 The study was approved by the Ethics Committee from the Hospital Central de las Fuerzas Armadas.

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489

490 **Informed consent**

491 The patient gave written informed consent

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785

786 **Figure Captions**

787 **Fig. 1.** Multiple sequence alignment of viral RNA-dependent RNA polymerase (RdRp). A. Complete RdRp
788 sequence alignment for Hepatitis E virus (HEV) reference genotypes (HEV1-HEV8), subtypes (HEV 3a-3m) and
789 HEV_C1_Uy. The black dot indicates the non-reported substitution V/I4A and the infrequent ones T/A392V,
790 V443A and Q484H. B. Sequence alignment for the RdRp catalytic site for Porcine Aichi virus (Kobuvirus),
791 Enterovirus A71, Coxsackie B3, B1, Rubella virus, Norwalk virus, Hepatitis A, Beet necrotic yellow vein virus
792 (BNYVV) and HEV_C1_Uy. The conserved motifs (D-x(4,5)-D) and GDD are highlighted in yellow and green,
793 respectively.

794

795 **Fig. 2.** Phylogenetic tree based on the RdRp catalytic site constructed by the Neighbour-Joining method with
796 Poisson model as the substitution model. HEV strain coming from the HEV-SOT recipient (♦) was compared to
797 other viral RdRp. Only bootstrap values >60% are shown.

798

799 **Fig. 3.** Secondary structure prediction for HEV_C1_Uy RdRp model employing PSIPRED. The Strand, Helix,
800 Coil distribution and its confidence of prediction is shown for each residue throughout the sequence.

801

802 **Fig. 4.** 3D Structural models of HEV RdRp obtained by I-TASSER. A. HEV_C1_Uy RdRp model. B. HEV
803 Arkell RdRp model. C. Structural alignment between HEV_C1_Uy (green) and HEV Arkell (red). D. Structural
804 alignment between HEV_C1_Uy (green) and the top threading template Porcine Aichi virus (Kobivirus) (blue)
805 (PDB: 6R1I). The finger's, thumb and palm domains are indicated in all the models. Graphs were obtained with
806 UCSF Chimera v. 1.8.

807

808 **Fig. 5.** Molecular docking analysis between HEV_C1_Uy RdRp and RBVT. A. 3D interaction of the HEV RdRp
809 with ligand RBVT in the defined finger's-palm domains binding pocket. Hydrogen-bonds interacting residues are
810 indicated in purple with single-letter amino acid code. Graphs were obtained with UCSF Chimera v. 1.8. B. 2D
811 diagram of the HEV RdRp-RBVT showing the residues forming hydrogen-bonds and the distance between atoms,
812 employing LigPlot+ v. 4.5.3.

813

814 **Fig. 6.** The RMSD and RMSF analysis of the MD trajectories. A – C. RMSD values expressed in Angstroms for
815 the whole duration of the MD simulation for the complexes containing Cinnamaldehyde, GTP, and RBVT
816 respectively. D – F. RMSF values expressed in Angstroms for the whole duration of the MD simulation and all
817 the protein residues of complexes containing Cinnamaldehyde, GTP, and RBVT respectively. RMSD values were
818 calculated for the protein backbone while RMSF values were calculated for C α atoms of each residue.