

Supplementary material. Emergence of progressive mutations in a hematologic patient with SARS-CoV-2 and prolonged viral replication.

Supplementary methods.

Microbiological studies

Reverse transcription polymerase chain reaction (RT-PCR) was performed using Cobas®6800 (Roche Diagnostic, Germany), which detects E and ORF1b genes. A positive result was defined when the cycle threshold (Ct) value of the E gene was ≤ 38 . All positive samples were tested for the presence of sub-genomic RNA (sgRNA) with primers and probes targeting sequence downstream of the start codons of the E gene, as described with a specific forward leader primer (Wölfel R. et al. *Nature* 2020. doi:10.1038/s41586-020-2196-x). sgRNA was performed using the SuperScript™ III Platinum™ One-Step qRT-PCR Kit (Invitrogen) in the thermocycler StepOne (Applied Biosystems). A positive result was defined when the Ct value of the E gene was ≤ 39 .

For viral culture, each sample was treated with a mixture of antibiotics (vancomycin and streptomycin) and antifungal (amphotericin B) for 30 minutes. After sample treatment, 300 μ l were inoculated into the VERO-E6 cell line and incubated at 37°C for a maximum of 10 days. A culture was considered positive when a characteristic CPE was observed. The CPE was confirmed to be caused by SARS-CoV-2 by indirect immunofluorescence using a specific monoclonal antibody AntiSARS-CoV-2 (CERTEST, Spain). The viral culture was considered negative in the absence of CPE 10 days after inoculation.

Sequencing methods

Whole genome amplification for NGS sequencing was performed at the National Center of Microbiology (ISCI). Sample cDNAs were obtained by means of a Sequence-Independent, Single-Primer-Amplification (SISPA) using the random primer FR26RV-N (5'-GCCGGAGCTCTGCAGATATCNNNNN-3') (Pérez-Sautu u. et al. *Emerg Microbes Infect* 2019. doi:10.1080/22221751.2019.1640587). Subsequently, cDNA products were amplified following ARTIC network's PCR protocol, with the primer pool version "ARTIC n-CoV-2019 v3" (Ewels P. et al. *Bioinformatics* 2016. doi:10.1093/bioinformatics/btw354). Libraries were performed following Nextera DNA library preparation kit instructions (Illumina, California, USA). These libraries were barcoded with unique dual indexes and then pooled and sequenced using a NextSeq Reagent kit 300 cycles (Illumina, California, USA) in a NextSeq sequencer (Illumina, California, USA). The sequences are available in the GISAID database (Shu Y. et al. *Eurosurveillance* 2017. doi:10.2807/1560-7917.ES.2017.22.13.30494).

To confirm Spike sequences, two PCRs targeting the S1 and S2 regions of the Spike protein were performed. The primers used in amplifying these regions included ARTIC pool primers 71L and 78R for S1 region and 77L and 84R primers for S2 region. Amplification conditions were: RT at 48°C - 20 min, 95°C - 5 min, followed by 45 cycles, 95°C - 15 s, 64°C - 2 min, 68°C - 30 s. Amplified products (~2490 bp S1 and ~2551 bp S2) were visualized performing an electrophoresis on a 1% agarose gel. Purified, amplified products of the expected size were double-strand sequenced by Sanger chain-termination method, using the BigDye Terminator v3.1 Cycle Sequencing Kit in an ABI PRISM 3700 DNA Analyzer (Applied Biosystems).

Quality control of the consensus sequences was based on the evaluation of the number of Ns in the consensus genome; number of ambiguous bases (Non A, T, C, or G); presence of mutations that only occur in this particular consensus genome; and description of frameshifts and premature stop codons. According to this quality control, the status was good in 10 of the 12 sequences analyzed, with a mean depth of coverage obtained between 4000-6000x.

With respect to the good-quality consensus sequences, 9 of these 12 samples had genome coverage superior to 90% after whole genome sequencing (WGS). In samples in which genome coverage was between 70-90%, genomic fragments negative by NGS methods were re-amplified and sequenced by Sanger. Spike genes in all samples were also sequenced by Sanger to confirm specific mutations.

Bioinformatic methods

Sequencing samples were analyzed for viral consensus genome reconstruction using the viralrecon pipeline (<https://github.com/nf-core/viralrecon>) written in Nextflow (<https://www.nextflow.io/>) in collaboration with the nf-core (<https://nf-co.re/>) community and the Bioinformatics Unit of the Institute of Health Carlos III (BU-ISCI) (<https://github.com/BU-ISCI>). In this pipeline, fastq files containing raw reads were first analyzed for quality using FastQC v0.11.9 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Raw reads were trimmed using fastp v.0.20.1 (Chen S. et al. *Bioinformatics* 2018. doi:10.1093/bioinformatics/bty560). The sliding window quality filtering approach was performed, scanning the read with a 4-base-wide sliding window and cutting 3' and 5' ends of the base when average quality per-base dropped below a Qphred33 of 30.

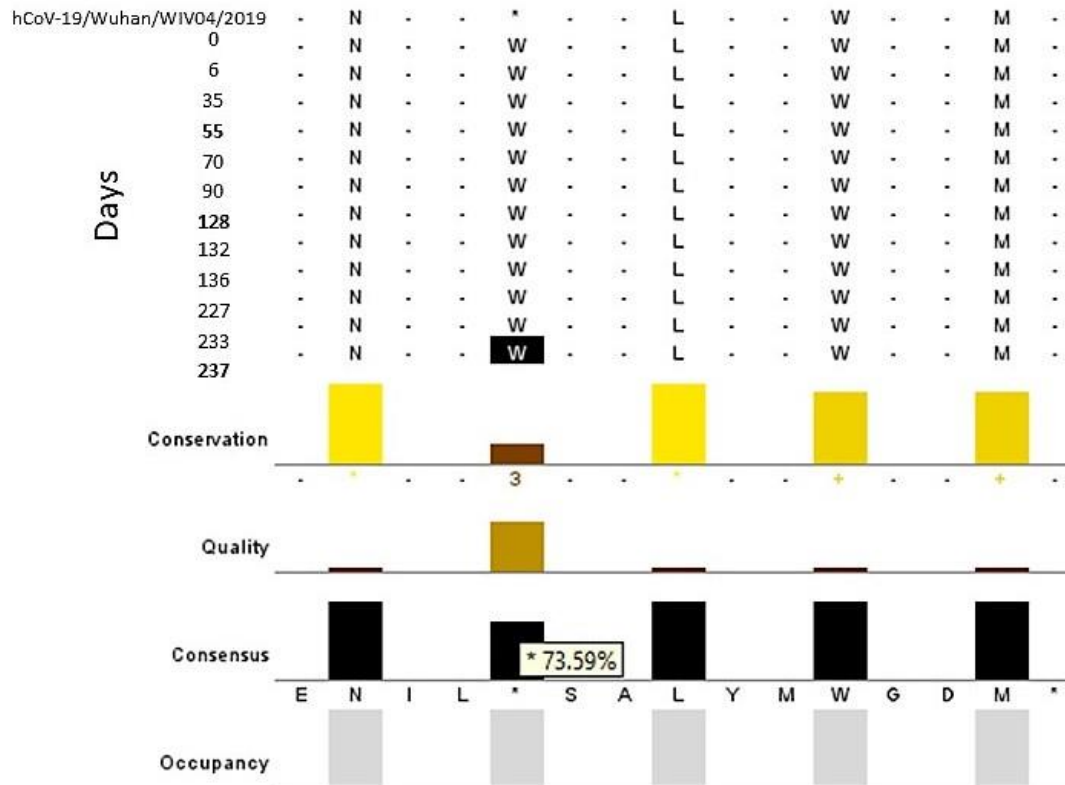
Reads shorter than 50 nucleotides and reads with more than 10% of read quality under Qphred 30 were removed. Additionally, poly-X sequenced were removed from read ends. Trimmed reads were mapped against the reference SARS-Cov2 genome (NC_045512.2) with bowtie2 v.2.3.5.1 (Langmead B. et al. *Nat Methods* 2012. doi:10.1038/nmeth.1923). Amplicon primers were then soft-clipped from mapping files using iVar v.1.2.2 (Grubaugh ND. et al. *Genome Biol* 2019. doi:10.1186/s13059-018-1618-7). Picard v.2.22.8 (<https://github.com/broadinstitute/picard>) and SAMtools v.1.9 (Li H. et al. *Bioinformatics* 2019. doi:10.1093/bioinformatics/btp352), were used to generate viral genome mapping stats. To obtain statistics about host genome content, kmer-based mapping of the trimmed reads against the GRCh38 NCBI human genome reference was performed with Kraken2 v.2.0.9beta (Wood DE. et al. *Genome Biol* 2019. doi:10.1186/s13059-019-1891-0).

Variant calling was done using VarScan2 v.2.4.4 (Koboldt DC. et al. *Bioinformatics* 2009. doi:10.1093/bioinformatics/btp373), which calls for low and high frequency variants from which variants with an allele frequency higher than 80 were kept to be included in consensus genome sequence. Both, variants included and not included in the consensus genome sequence were annotated using SnpEff v.4.5covid19 (Cingolani P. et al. *Fly (Austin)* 2012. doi:10.4161/fly.19695), and SnpSift v.4.3.1t (Cingolani P. et al. *Front Genet* 2012. doi:10.3389/fgene.2012.00035). Finally, bedtools v2.29.2 (Quinlan AR. et al. *Bioinformatics* 2010. doi:10.1093/bioinformatics/btq033) was used to obtain the viral genome consensus with the filtered variants and mask genomic regions with coverage values lower than 10X. Final summary reports were created using MultiQC v.1.9 (Ewels P. et al. *Bioinformatics* 2016. doi:10.1093/bioinformatics/btw354).

The variant calling files (vcf) obtained with VarScan2 containing all of the variants (low and high frequency) were analyzed with Bcftools v.1.10.2 to merge all of the samples and select the allele frequency field (Danecek P. et al. *Gigascience* 2021. doi:10.1093/gigascience/giab008). Annotation information from SnpEff was then added to the variants. In order to create ad-hoc tables and allele frequency evolution plots, the resulting table underwent further processing with R v.3.6.1.

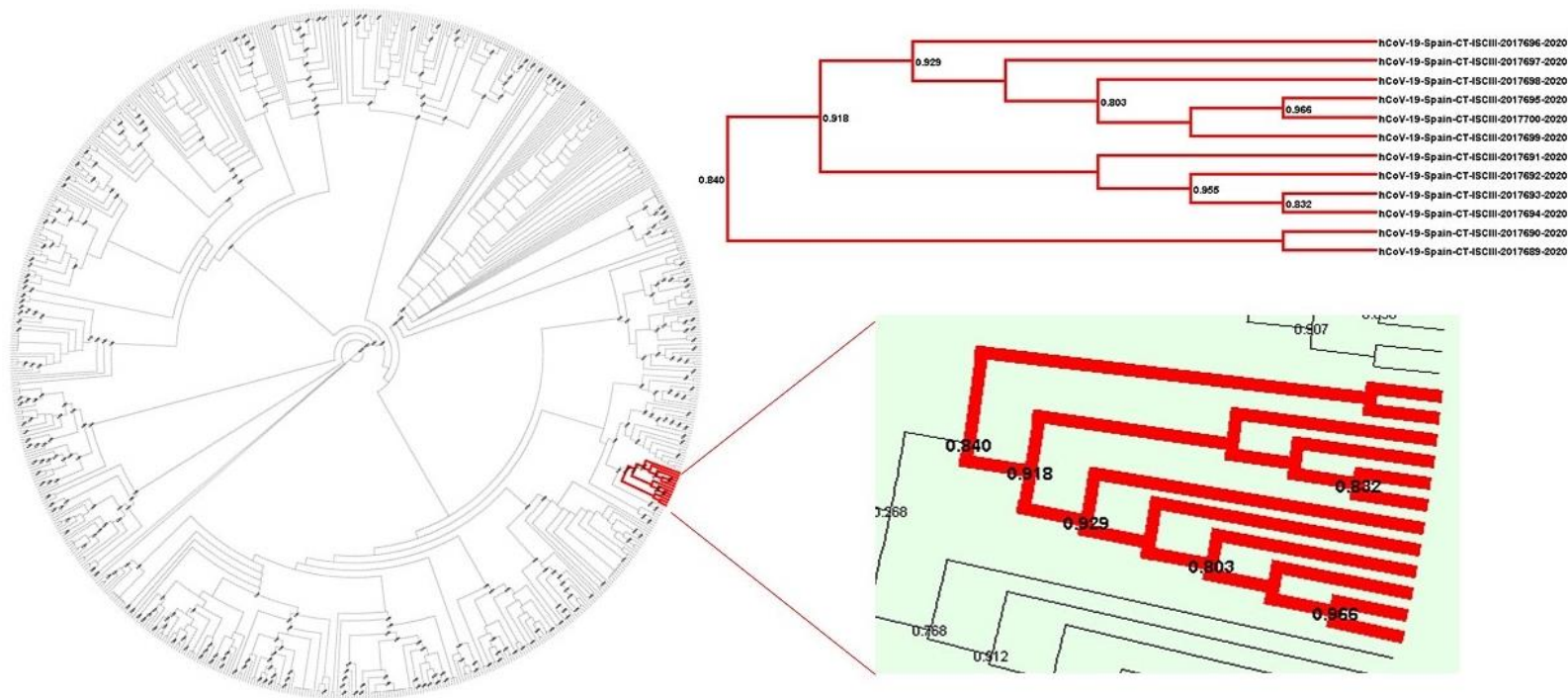
Supplementary results.

Supplementary Figure S1. Aminoacidic alignment chart of the patient's sequences with all the B.1 complete sequences published in Spain from March to December 2020 (n= 6761 sequences).



Legend. The presence of a Tryptophan in the position 6668 occurred 27% of the times.

Supplementary Figure S2. Phylogenetic tree based on the analysis of B.1 complete sequences published in GISAID from Spain from March to December 2020 (n= 741 sequences).



Legend. Trees were estimated with FastTreeMP software by using the Maximum Likelihood method on Generalized Time-Reversible model. A bootstrap test was replicated for 1000 times. Numbers represent percentage bootstrap support. GISAID accession numbers for the sequences included in the tree are listed in Supplementary Table S1.

Supplementary Table S1. GISAID accession numbers from the sequences used in the phylogenetic analysis.

Sequence ID	GISAID accession number
hCoV-19-Spain-VC-FISABIO-187-2020	EPI-ISL-436282
hCoV-19-Spain-VC-FISABIO-192-2020	EPI-ISL-436287
hCoV-19-Spain-VC-FISABIO-202-2020	EPI-ISL-436297
hCoV-19-Spain-AR-IBV-2451-2020	EPI-ISL-468770
hCoV-19-Spain-AR-IBV-2487-2020	EPI-ISL-468771
hCoV-19-Spain-AR-IBV-2414-2020	EPI-ISL-468772
hCoV-19-Spain-AR-IBV-2475-2020	EPI-ISL-468775
hCoV-19-Spain-AS-IBV-98006633-2020	EPI-ISL-654625
hCoV-19-Spain-AS-IBV-98006624-2020	EPI-ISL-654627
hCoV-19-Spain-AS-IBV-98006651-2020	EPI-ISL-654629
hCoV-19-Spain-AS-IBV-98006628-2020	EPI-ISL-654631
hCoV-19-Spain-AS-IBV-98006646-2020	EPI-ISL-654633
hCoV-19-Spain-VC-FISABIO-106-2020	EPI-ISL-436201
hCoV-19-Spain-VC-FISABIO-108-2020	EPI-ISL-436203
hCoV-19-Spain-VC-FISABIO-110-2020	EPI-ISL-436205
hCoV-19-Spain-VC-FISABIO-112-2020	EPI-ISL-436207
hCoV-19-Spain-VC-FISABIO-114-2020	EPI-ISL-436209
hCoV-19-Spain-VC-FISABIO-129-2020	EPI-ISL-436224
hCoV-19-Spain-VC-FISABIO-134-2020	EPI-ISL-436229
hCoV-19-Spain-VC-FISABIO-135-2020	EPI-ISL-436230
hCoV-19-Spain-VC-FISABIO-142-2020	EPI-ISL-436237
hCoV-19-Spain-VC-FISABIO-145-2020	EPI-ISL-436240
hCoV-19-Spain-VC-FISABIO-147-2020	EPI-ISL-436242
hCoV-19-Spain-VC-FISABIO-149-2020	EPI-ISL-436244
hCoV-19-Spain-VC-FISABIO-150-2020	EPI-ISL-436245
hCoV-19-Spain-VC-FISABIO-152-2020	EPI-ISL-436247
hCoV-19-Spain-VC-FISABIO-155-2020	EPI-ISL-436250
hCoV-19-Spain-VC-FISABIO-157-2020	EPI-ISL-436252
hCoV-19-Spain-VC-FISABIO-163-2020	EPI-ISL-436258
hCoV-19-Spain-VC-FISABIO-166-2020	EPI-ISL-436261
hCoV-19-Spain-VC-FISABIO-167-2020	EPI-ISL-436262
hCoV-19-Spain-VC-FISABIO-172-2020	EPI-ISL-436267
hCoV-19-Spain-VC-FISABIO-173-2020	EPI-ISL-436268
hCoV-19-Spain-VC-FISABIO-176-2020	EPI-ISL-436271
hCoV-19-Spain-VC-FISABIO-177-2020	EPI-ISL-436272
hCoV-19-Spain-VC-FISABIO-156-2020	EPI-ISL-436251
hCoV-19-Spain-AR-IBV-2387-2020	EPI-ISL-468840
hCoV-19-Spain-AR-IBV-2419-2020	EPI-ISL-468842
hCoV-19-Spain-AR-IBV-2430-2020	EPI-ISL-468843
hCoV-19-Spain-AR-IBV-2396-2020	EPI-ISL-468851
hCoV-19-Spain-AR-IBV-2462-2020	EPI-ISL-468854
hCoV-19-Spain-NC-CHN-01000278-2020	EPI-ISL-1382967
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hCoV-19-Spain-NC-CHN-01000282-2020	EPI-ISL-1382970
hCoV-19-Spain-NC-CHN-01000285-2020	EPI-ISL-1382972
hCoV-19-Spain-NC-CHN-01000286-2020	EPI-ISL-1382973
hCoV-19-Spain-NC-CHN-01000288-2020	EPI-ISL-1382975
hCoV-19-Spain-NC-CHN-01000289-2020	EPI-ISL-1382976
hCoV-19-Spain-NC-CHN-01000291-2020	EPI-ISL-1382978
hCoV-19-Spain-NC-CHN-01000293-2020	EPI-ISL-1382980
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hCoV-19-Spain-NC-CHN-01000301-2020	EPI-ISL-1382987
hCoV-19-Spain-NC-CHN-01000302-2020	EPI-ISL-1382988
hCoV-19-Spain-NC-CHN-01000303-2020	EPI-ISL-1382989
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hCoV-19-Spain-NC-CHN-01000314-2020	EPI-ISL-1382999
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hCoV-19-Spain-AR-IBV-2422-2020	EPI-ISL-468815
hCoV-19-Spain-AR-IBV-2412-2020	EPI-ISL-468858
hCoV-19-Spain-AR-IBV-2423-2020	EPI-ISL-468859
hCoV-19-Spain-PV-IBV-2077-2020	EPI-ISL-468872
hCoV-19-Spain-PV-IBV-2130-2020	EPI-ISL-468878
hCoV-19-Spain-RI-IBV-99010924-2020	EPI-ISL-732763
hCoV-19-Spain-NC-CHN-01000307-2020	EPI-ISL-1382909
hCoV-19-Spain-NC-CHN-01000239-2020	EPI-ISL-1382910
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hCoV-19-Spain-NC-CHN-01000165-2020	EPI-ISL-1382914
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hCoV-19-Spain-NC-CHN-01000162-2020	EPI-ISL-1382927
hCoV-19-Spain-NC-CHN-01000245-2020	EPI-ISL-1382943
hCoV-19-Spain-NC-CHN-01000253-2020	EPI-ISL-1382945
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hCoV-19-Spain-NC-CHN-01000256-2020	EPI-ISL-1382947
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hCoV-19-Spain-NC-CHN-01000290-2020	EPI-ISL-1382977
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hCoV-19-Spain-VC-FISABIO-233-2020	EPI-ISL-436328
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hCoV-19-Spain-VC-FISABIO-291-2020	EPI-ISL-436386
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hCoV-19-Spain-MD-IBV-003708-2020	EPI-ISL-467199
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hCoV-19-Spain-PV-IBV-004200-2020	EPI-ISL-510485
hCoV-19-Spain-PV-IBV-004204-2020	EPI-ISL-510489
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hCoV-19-Spain-VC-IBV-98004548-2020	EPI-ISL-796111
hCoV-19-Spain-VC-IBV-98004556-2020	EPI-ISL-796114
hCoV-19-Spain-VC-IBV-98004572-2020	EPI-ISL-796117

hCoV-19-Spain-VC-IBV-98004583-2020	EPI-ISL-796119
hCoV-19-Spain-VC-IBV-98004586-2020	EPI-ISL-796122
hCoV-19-Spain-MD-IBV-99013151-2020	EPI-ISL-831040
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hCoV-19-Spain-MD-IBV-99013163-2020	EPI-ISL-831050
hCoV-19-Spain-MD-IBV-99013164-2020	EPI-ISL-831051
hCoV-19-Spain-MD-IBV-99013166-2020	EPI-ISL-831053
hCoV-19-Spain-MD-IBV-99013168-2020	EPI-ISL-831054
hCoV-19-Spain-MD-IBV-99013169-2020	EPI-ISL-831055
hCoV-19-Spain-MD-IBV-99013170-2020	EPI-ISL-831056
hCoV-19-Spain-MD-IBV-99013171-2020	EPI-ISL-831057
hCoV-19-Spain-MD-IBV-99013172-2020	EPI-ISL-831058
hCoV-19-Spain-MD-IBV-99013173-2020	EPI-ISL-831059
hCoV-19-Spain-MD-IBV-99013174-2020	EPI-ISL-831060
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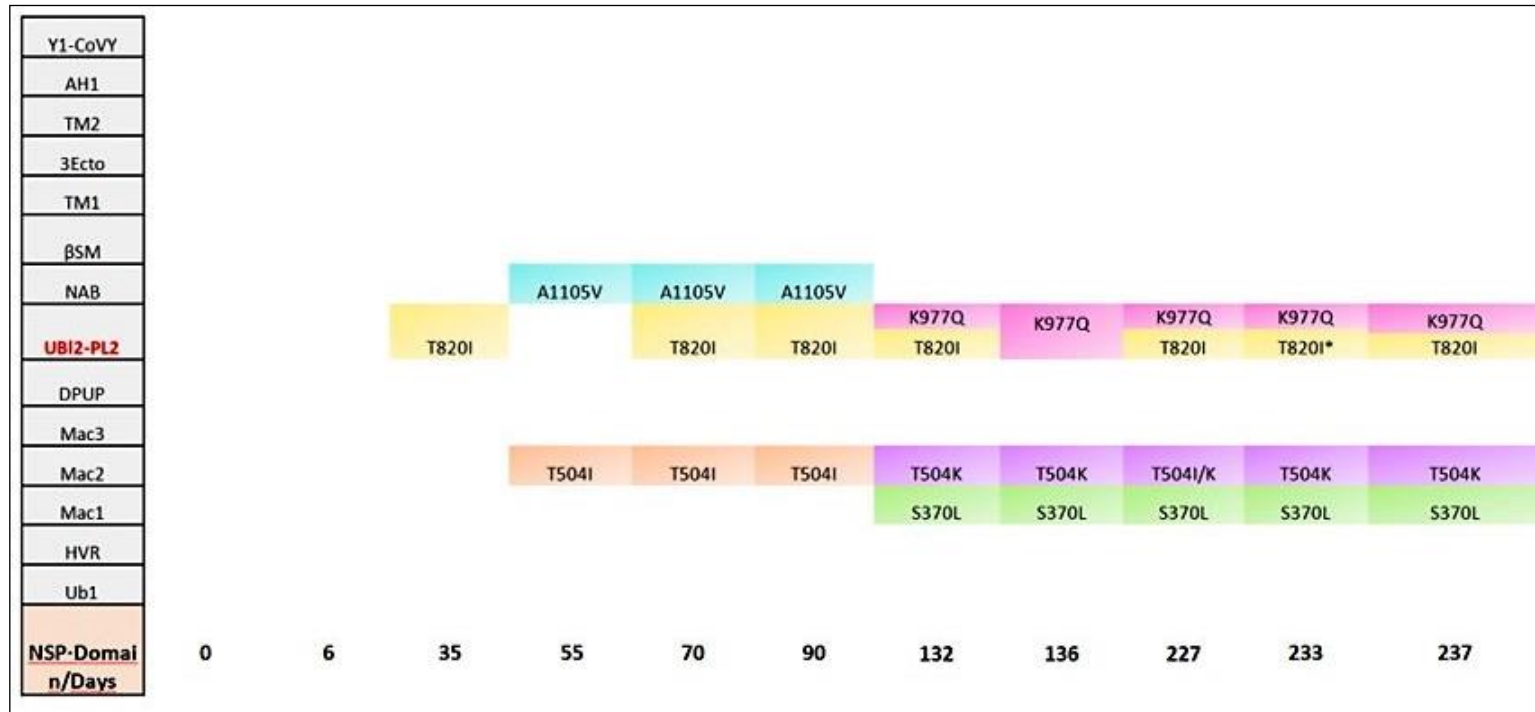
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NC-045512.2 Severe acute respiratory syndrome coronavirus 2-isolate-Wuhan-Hu-1-complete-genome: GenBank accession number: NC_045512	

Supplementary Table S2. Summary of 12 samples of SARS-Cov-2 genetic study.

Sample number	Date of collection	Days since first sampling	Nucleotide mutations*	Amino acid mutations*	Completed by Sanger	Lineage
1	2020-03-24	0	7	2		B.1
2	2020-03-30	6	8	3		B.1
3	2020-04-28	35	8	3		B.1
4	2020-05-18	55	11	5	Yes	B.1
5	2020-06-02	70	12	6		B.1
6	2020-06-22	90	14	8		B.1
7	2020-07-30	128	20	15		B.1
8	2020-08-03	132	18	12	Yes	B.1
9	2020-08-07	136	16	10		B.1
10	2020-11-06	227	17	10		B.1
11	2020-11-12	233	21	13	Yes	B.1
12	2020-11-16	237	29	22		B.1

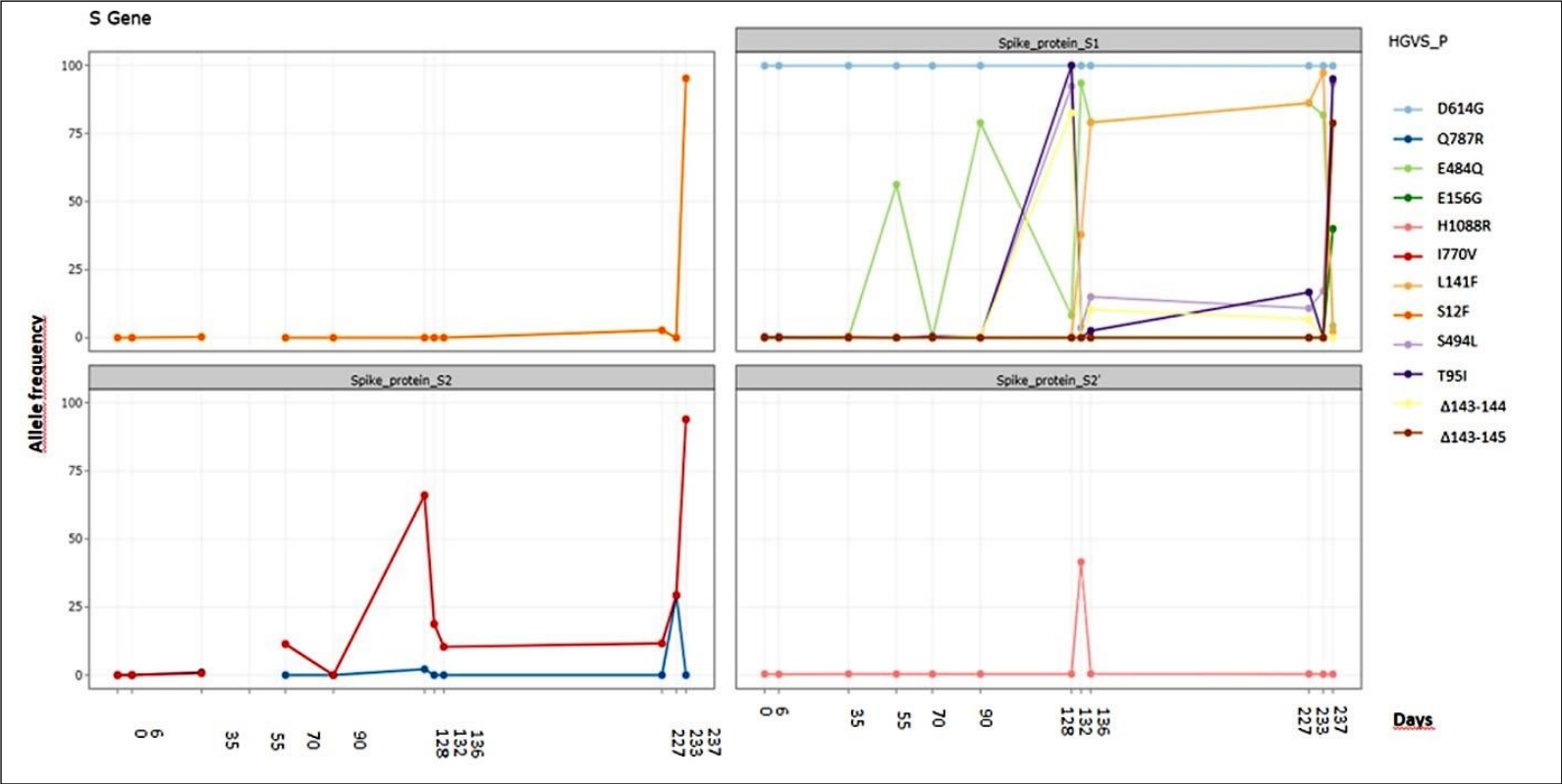
* Compared with the reference SARS-CoV-2 Wuhan-1 (GenBank accession number: NC 045512).

Supplementary Figure S3. Description of the different mutations in nsp3 dominions over time.



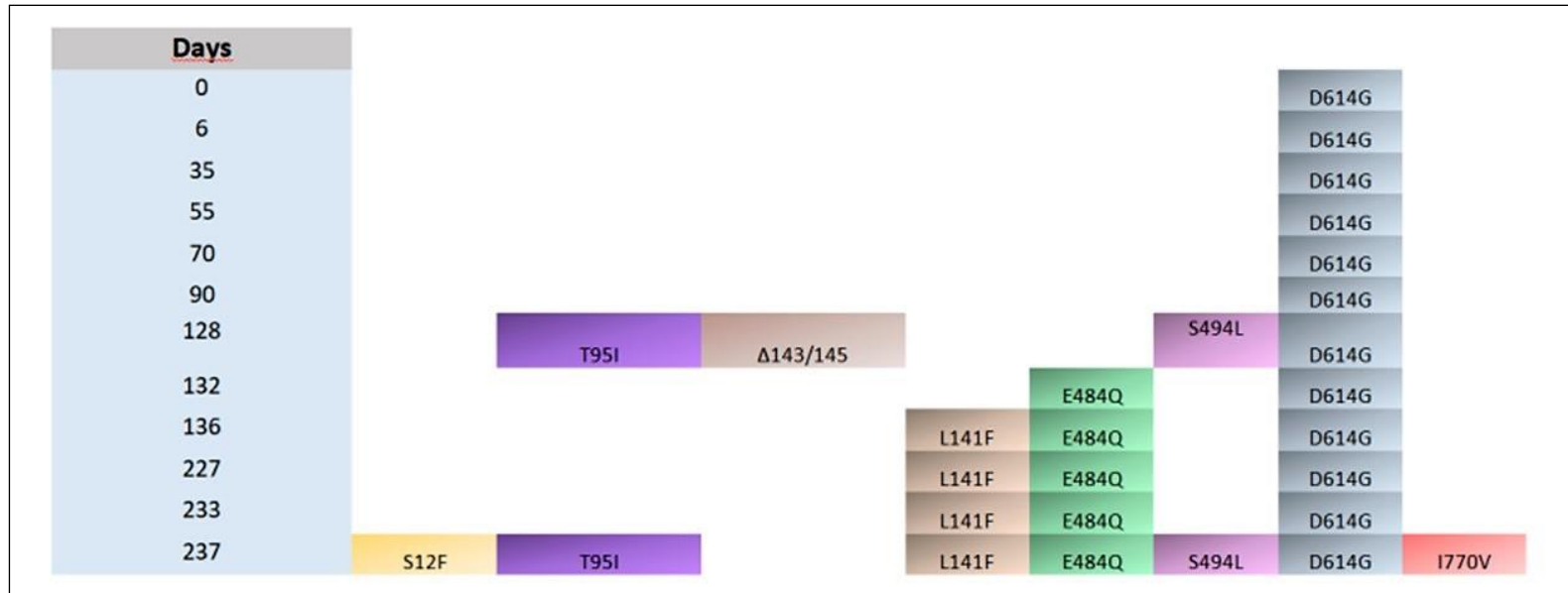
Legend. The X axis represents the days since the first positive sample. Minor variants and amino acid changes in nsp3 appeared after day 35. According to the consensus sequences, 6 amino acids changes were described over time in nsp3, mainly in Mac1, Mac 2 and UBI2-PI2pro domains. The amino acid change S370L in Mac1 was detected in samples from day 132 to day 237. Mac2 presented two amino acid non-conservative changes in the position 504 of the protein. From day 55 to day 90 a change from tryptophan to interleucine was evidenced, and this interleucine that turned into a lysine from day 132 to 237. Three changes in the UBI2-PI2pro domain were evidenced: T820I, K977Q and E746A.

Supplementary Figure S4. The most important mutations documented in the consensus sequences of the Spike protein over time.



Legend. Time evolution of the most relevant mutations in the Spike protein. Variants showed a frequency from 0% to 100% (Y axis) over the different sampling dates (X axis). Amino acid substitutions are indicated by points using the same coloring associated to each name in the legend.

Supplementary Figure S5. Amino acid changes in the Spike protein observed in the last sample.



Legend. Description of the different mutations characterized in the Spike's consensus sequences over time. The X axis represents the days since the first positive sample. According to the consensus sequences, 8 amino acids changes were described over time in the Spike protein.