Coronavirus SARS-CoV-2: Analysis of subgenomic mRNA transcription, 3CLpro and PL2pro protease cleavage sites and protein synthesis

Corresponding author: Miguel Ramos-Pascual

Abstract

Coronaviruses have recently caused world-wide severe outbreaks: SARS (Severe Acute Respiratory Syndrome) in 2002 and MERS (Middle-East Respiratory Syndrome) in 2012. At the end of 2019, a new coronavirus outbreak appeared in Wuhan (China) seafood market as the first focus of infection, becoming a pandemic in 2020, spreading mainly into Europe and Asia [Zu et al 2020]. Although the virus family is well-known and symptoms are similar to other coronaviruses (fever, pneumonia, small pleural effusions), this specific virus type presents considerable differences, as higher transmission and mortality rates, being a challenge for diagnostic methods, treatments and vaccines.

Coronavirus.pro (SARS-CoV-2) App is a module of Virus.pro, a C++ application which simulates Coronavirus replication cycle. This software identifies virus types in short times and provides FASTA files of virus proteins, a list of RNA sequences (regulatory, packaging, transcription and translation) and secondary structures (stem-loops, helices, palindromes, mirrors), once the virus genome has been sequenced. The code is supported by other bio-informatics tools, such as Vienna RNA package, Varna software and ClustalW2.

Coronavirus.pro has identified 2019-nCoV virus as a beta-coronavirus more close related to SARS type than to MERS. However, it presents significant differences, such as the spike glycoprotein precursor, characteristic of this virus type, and the increased number of transcription regulating sequences (TRS), producing more subgenomic mRNAs and synthesizing more fusion proteins than SARS/MERS. This could be related with those severe health effects (toxicity) on host patients than other coronaviruses.

The software has identified a list of structural, non-structural and accessory proteins in 2019-nCoV virus genome similar to SARS and MERS. It has found also several ORF encoding some accessory proteins with unknown TRS (i.e. AP3b, AP9b, AP11, AP12 and AP14a/b). Furthermore, there is a subgenomic mRNA, the shortest with 374bp, which translates no proteins, specific only of SARS type virus. Finally, there are some accessory proteins AP2 in SARS/MERS and AP2a/b in 2019-nCoV, encoded before ORF1.2 and ORF1.4 respectively, which have not been previously reported.

2019-nCoV protein sequences have been compared with those from SARS and MERS. As 3CLpro (nsp5) and RdRp (nsp12) have >90% similarities with SARS, some antiviral drugs effective with SARS coronavirus, such as protease inhibitors or RNA-dependent RNA polymerase inhibitors could be also effective to this virus type. Nevertheless, further comparisons would be required, including other types of estimators.

These results are useful as a first step with other bio-informatics and pharmacological tools in order to develop diagnostic methods (real time RT-PCR or ELISA tests), new vaccines or antiviral drugs, which avoid virus replication in any stage: fusion inhibitors, RdRp inhibitors and PL2pro/3CLpro protease inhibitors.

Keywords: SARS-CoV, MERS-CoV, 2019-nCoV, Coronavirus, virus proteins, protease cleavage sites
1. Introduction

Coronaviruses (CoVs) are specific viruses that cause diseases in mammals and birds, including humans, with symptoms such as enteritis in bats, mice and pigs and upper respiratory malfunctions and potentially lethal respiratory infections in humans [Fehr and Perlman 2015]. A large variety of coronaviruses have been previously studied and analyzed. These viruses are responsible in a 2-10% of common cold in immunocompetent individuals (i.e. 229E, OC43E, NL63 and HKU1 types). However, other types can cause severe respiratory syndromes, such as SARS-CoV (Severe Acute Respiratory Syndrome Coronavirus) that caused an epidemics in 2002-2003, with origin on Guangdong (China) [Vijayanand et al 2004] and MERS-CoV (Middle Eastern Respiratory Syndrome Coronavirus) expanded in 2014-2015 through Saudi Arabia into Egypt, Oman and Qatar, from bats to dromedary camels, as the source of infection in humans [Aleanizy et al 2017].

At the end of 2019, a new SARS outbreak appeared in Wuhan (China) seafood market as the first focus of infection, becoming a pandemics in 2020 and spreading mainly into Europe and Asia, declaring general state of alarm in several countries, as Spain and Italy [Liao et al 2020, Zu et al 2020, Giovanetti et al 2020].

Coronavirus infections have normally low case fatality rates, with symptoms more severe than common cold, affecting mainly respiratory tract (cilia epithelium of the trachea, nasal mucosa and alveolar cells of the lung). Although the virus family is well-known and symptoms are similar to other coronavirus (fever, pneumonia, small pleural effusions), this specific type of virus presents considerable differences, as a higher infection/transmission and mortality rate, being a challenge for disease protection, prevention, diagnostic methods, vaccines and treatments [Wu et al 2020, Zhu et al 2020].

Virus pharmacology is based on preventive actions (vector-based or RBD-based vaccines), diagnostic methods (real time RT-PCR or ELISA tests) and antiviral drugs. SARS-CoV and MERS-CoV epidemics have expanded the use of several drugs, specially virus cycle inhibitors against coronaviruses (fusion inhibitors, RdRp inhibitors or PL2pro/3CLpro protease inhibitors) [Li G and De Clercq 2019, Raoult et al 2020].

In order to develop these methods, virus replication cycle must be simulated through computerized tools, specially for this virus family, with a complex replication cycle. Coronaviruses synthesize in a first stage a viral RNA-dependent RNA polymerase (RdRp) and multiple proteases, transcribes several subgenomic mRNAs and translates them progressively into viral proteins through several ribosomal pathways: (-1) programmed frameshift, leaky scanning and internal ribosome entry site (IRES) [Plant et al 2005]. Some mRNAs include genes encoding large polypeptide chains, which are cleaved through 3CLpro and PL2pro proteases, producing non-structural proteins as enzymes for catalyzing assembly and packaging of new viruses [Sawicki et al 2007, Fehr and Perlman 2015, Oxford et al. 2016].

Coronavirus.pro (2019-nCoV) App is a C++ code which simulates Coronavirus replication cycle. This software identifies virus types in short times and provides FASTA files of virus proteins, a list of RNA sequences (regulatory, packaging, transcription and translation) and secondary structures (stem-loops, helixes, palindromes, mirrors), once the virus genome has been sequenced [Ramos-Pascual 2019]. The code is supported by other RNA analysis tools, such as Vienna RNA package and Varna software [Gruver et al 2008, Darty et al 2009]. These results are useful as a first step with other bioinformatics and pharmacological tools in order to develop diagnostic methods, new vaccines and antiviral drugs.
2. The Coronavirus: classification, structure, genome and virus cycle

2.1 Classification

Coronaviruses (CoVs) are part of the order Nidovirales, from the family Coronaviridae and formed by several subtypes: Alpha-, beta-, gamma- and delta-coronavirus. Virus 229E and OC43, the first of being isolated and responsible of common cold, belong to alpha-coronavirus group I, while SARS and MERS are beta-coronavirus.

2.2 Structure of the virion

Coronaviruses have diameters from 100 to 160 μm with very large heavily glycosylated spikes (S) of 200kDa and 20 μm, placed around virus membrane as a crown, hence their name, in a trimer configuration (fig. 1).

Viral RNA genome is encapsulated in a helicoidal nucleocapsid phosphoprotein (N), known also as ribonucleoprotein (RNP), and enveloped into a virus particle with different membrane (M) and envelope glycoproteins (E).

Some coronaviruses include also a hemagglutinin-acetyltransferase glycoprotein (HE) in the outer membrane. HE helps during attachment, destroying certain sialic acid receptors in host-cell surface. Not all strains demonstrated hemagglutination, as observed only in beta-coronaviruses subgroup 2a (HCoV-HKU1, MHV) and toroviruses (BToV) [Brian et al. 1995, de Groot 2006]

![Fig. 1 - Structure of a general Coronavirus virion particle](image)

2.3 Virus genome

Coronavirus genome is a type of positive single-stranded RNA of approximately 30kb, the largest of RNA viruses, 5’capped, 3’polyadenylated and infectious. This Poly(A) tail allows coronaviruses direct translation after infection without needing an intermediate transcription stage.

Transcription initiation is regulated in coronaviruses by several types of consensus transcription regulating sequences: TRS1-L, 5’-cuaaac-3’, TRS2-L, 5’-acgaac-3’ and merged into TRS3-L, 5’-cuaacgaac-3’. These multiple TRS give place to several subgenomic polycistronic mRNAs, encoding structural, non-structural and accessory proteins. In case of some coronaviruses (i.e. MERS-CoV), transcription starts mainly in TRS2-L, while other coronaviruses start transcription indistinctly in all TRS, in a way of regulating protein frequencies [Sethna et al 1989, Irigoyen et al 2016].

Coronavirus genome includes multiple open-reading frames (ORF) containing genes which are transcribed by several transcription regulating sequences (TRS). Genes encoding non-structural proteins are placed at
5’ UTR (ORF1.1, ORF1.2 ...), whereas at 3’ UTR are genes for structural (N, M, E and S). These genes are interspaced with several accessory genes, encoding accessory proteins (AP), characteristics in number of each virus type. Some of these AP are not essential for in vitro or in vivo replication.

As transcription starts at different TRS in each subgenomic mRNA, the number of ORF genes is variable on virus type, and therefore the number of polypeptide chains. This produces different frequencies of non-structural proteins during virus cycle. For example, SARS produces ORF1.1 and ORF1.2 genes, whereas 2019-nCoV, produces ORF1.1 to ORF1.6 genes, synthesizing several groups of fusion proteins [van Boheemen et al. 2012]. A (-1) programmed slippery ribosome frameshift is placed approximately in the middle of ORF1 genes, then translated into polypeptides pp1a and pp1ab [Dinman 2012, Bock et al 2019]. Furthermore, Coronaviruses uses a leaky scanning mechanism (shunting) to synthesize proteins from overlapping ORF, translating different proteins from the same mRNA [Nakagawa et 2016].

Surface glycosyllabeled Spike (S) is processed in some coronaviruses from a proteolytic cleavage of a spike precursor [Belouzard et al 2009]. The number of spike precursors is characteristic of each coronavirus. For example, in the case of SARS-CoV, two spike precursors (Sp1 and Sp2) are proteolytically cleaved, producing two surface glycosyllabeled spikes (S1 an S2) and a protease fragment (S0).

Figure 2 shows a scheme of the main genes in Coronavirus family, including ORF1.1 with a -1 slippery ribosome frameshift. Genes transcribed from different TRS are placed in another line. Figure 3 presents a scheme of ORF1.1 gene and non-structural proteins (nsp1 to nsp16), including accessory protein AP2. Tables 1 and 2 summarize main genes and proteins of SARS-CoV and MERS-CoV coronaviruses.
Fig. 2 – Scheme of the genes in viral genomes from Coronavirus family: non-structural proteins (white), structural (green), accessory (blue) and other ORF (grey). Fusion proteins and subgenomic mRNAs are not depicted.

Fig. 3 – Scheme of the ORF1.1 gene and description of the non-structural proteins (nsp1 to nsp16) of SARS-CoV (SWISS Model) Accessory protein AP2 has been included
Table 1 - Summary of the main genes and characteristics of SARS-CoV [Xu et al 2003, Liu et al 2014] and MERS-CoV [Li et al 2019]

| Type                        | Coding Genes | Protein | Description                                                                 |
|-----------------------------|--------------|---------|-----------------------------------------------------------------------------|
| Non-structural              | ORF 1.1      | pp1a    | Polyprotein 1a                                                              |
|                             |              | pp1ab   | Polyprotein 1ab, [-1] PRF                                                   |
| ORF 1.2                     |              | AP2     | Accessory protein AP2, unknown                                              |
|                             |              | pp1a    | Polyprotein 1a                                                              |
|                             |              | pp1ab   | Polyprotein 1ab, [-1] PRF                                                   |
| ORF 2                       |              | Sp(S)   | Surface Glycosylated Spike precursor (Sp)                                   |
|                             |              | S       | Surface Glycosylated Spike (S)                                              |
|                             |              | S0      | Spike protease fragment (S0)                                                |
| ORF3a                       |              | AP3a    | Viral pathogenesis, apoptosis induction, cell cycle arrest, modulation of NF-kb-mediated inflammation |
| ORF3b                       |              | AP3b(b) | IRES translation, viral pathogenesis, not required for SARS-CoV replication |
| ORF4                        |              | E       | Envelope membrane                                                           |
| ORF5                        |              | M       | Transmembrane glycoprotein                                                  |
|                             |              | AP5     | Unknown, only MERS                                                          |
| ORF6                        |              | AP6     | Type I IFN production and signaling inhibition, only SARS                  |
| ORF7                        |              | AP7a/b  | Viral pathogenesis, apoptosis induction, cell cycle arrest, modulation of NF-kb-mediated inflammation |
| ORF8                        |              | AP8a/b  | DMV formation, complex with nsp3                                             |
|                             |              | unknown | ADP-ribosyltransferase 1-phosphatase (PL2pro (papain-like protease 2))      |
| ORF9                        |              | N       | Nucleocapsid phosphoprotein                                                 |
| ORF9b                       |              | AP9b(b) | Viral pathogenesis, apoptosis induction, cell cycle arrest, modulation of NF-kb-mediated inflammation, named AP8b in MERS |
| ORF11                       |              | AP11(b) | Unknown, only SARS                                                          |
| ORF14                       |              | AP14a/b | unknown                                                                      |

(a) - Spike precursors length and number depends on virus type (Sp=S+S0) (b) TRS unknown

Table 2 - Description of non-structural proteins (Polyprotein pp1ab) of SARS and MERS coronavirus [Chen et al 2020]

| Protease | Protein | Description                                                                 |
|----------|---------|-----------------------------------------------------------------------------|
| PL2pro   | nsp1    | Leader protein, suppress antiviral host response, promotes degradation of host mRNAs, inhibiting IFN signaling |
|          | nsp2    | unknown                                                                     |
|          | nsp3    | ADP-ribosyltransferase 1-phosphatase, PL2pro (papain-like protease 2)       |
|          | nsp3a   | unknown                                                                     |
|          | nsp3b   | unknown                                                                     |
|          | nsp4    | DMV formation, complex with nsp3                                             |
|          | nsp5    | 3C-like (3CLpro), Mpro, polypeptides cleaving                                |
|          | nsp6    | Restricting autophagosome expansion, DMV formation                           |
|          | nsp7    | Cofactor with nsp8 and nsp12                                                |
|          | nsp8a   | DNA primase, cofactor with nsp7 and nsp12                                    |
|          | nsp8b   | unknown                                                                     |
|          | nsp9    | Dimerization and RNA/DNA binding activity                                    |
|          | nsp10   | interacts with nsp14 and nsp16 [Bouvet et al 2010,2012]                     |
|          | nsp11   | Short peptide at pp1a end                                                   |
|          | nsp12   | RNA-dependent RNA polymerase (RdRp)                                         |
|          | nsp13a  | Helicase, NTPase nucleoside 5’ triphosphatase (2D, NTPase/HEL)              |
|          | nsp13b  | 3’-to-5’ exoribonuclease (nuclease ExoN homolog)                            |
|          | nsp14   | Endoribonuclease (endoRNase), evasion of dsRNA sensors                      |
|          | nsp15   | 5'-adenosylmethylamine-dependent ribose 2’-O- methyltransferase (2’-O-MT)   |
|          | nsp16   | unknown                                                                     |
2.4 Virus replication cycle

As other viruses, Coronavirus employs glycosillabed spikes placed in the outer surface to attach specific receptors of host cells (i.e. APN/ACE2/DPP4 receptor). Specially, betacoronaviruses attach to angiotensin-converting enzyme 2 (ACE2) receptor, a membrane protein expressed mainly in the surface of epithelial cells of the pulmonary alveolus [Jia et al 2005]. Once attachment is carried out, viral transmembrane is fused through an endocytotic pathway and viral RNA is released into the cell cytoplasm [Wang et al 2008]. Viral RNA genome is positive stranded, 5' capped and 3'polyadenylated, therefore it can be directly translated into proteins by host-cell ribosomes. Specifically, ORF1 gene contains several non-structural proteins in a polypeptide complex, that once translated, is are catalytically autoprocessed by 3CL/2PL proteases, and assembled into a replicase-transcriptase complex with RNA-dependent RNA activity (RdRp). At this point, several subgenomic mRNAs are produced by transcription and translated into structural (N, M, E and S), non-structural and accessory proteins (AP), assembling new virions. These virus particles are formed on smooth-walled vesicles located between the ER and the Golgi, named as ERGIC (Endoplasmic Reticulum Golgi Intermediate Compartment). Once these vesicles fuse with the plasma outer membrane, virions are released to continue infection (see fig 4).

![Figure 4 – Scheme of a Coronavirus cycle replication:](http://app.biorender.io)
3. Coronavirus.pro

3.1 Description

Coronavirus.pro is a module of Virus.pro, a C++ software application developed in modules that simulate mainly RNA and DNA virus replication cycles: Ebola, HIV-1, HCV (Hepatitis C), CoV, HSV1 (Human Herpes Virus), PV1 (Poliovirus 1, Mahoney). The software reproduces several virus cycle replication stages, from attachment and fusion to virion exit from host-cell, focusing into more complex stages, such as subgenomic mRNAs translation, protein synthesis and protease catalytic processing (see fig. 5 and 6).

Virus.pro contains a set of RNA/DNA databases and protein databases to scan viral genome and protein sequences for recognized motifs, reconstruct secondary structures (helixes, stem-loops, palindromes, mirrors) and identify RNA-protein interaction regions. The software is supported with other applications, as Vienna RNA package, for bracket-dot notation and Varna for plotting (see fig. 7) [Gruber et al 2008, Darty et al 2009]. The code contains also machine-learning algorithms, in which new virus, RNA/DNA sequences and proteins can be included to the internal databases to future identifications and analysis. The software has been validated with other bio-informatic tools, as Blastp or Swiss-Model [Altschul et al 1990, Camacho et al 2008, Waterhouse et al 2018, Ramos-Pascual 2019].

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Virus.pro

Analysis of virus cycle replication:

[1] CoV (SARS, MERS)
[2] Ebola
[3] HIV-1
[4] HCV (Hepatitis C virus)
[5] HSV1 (Human Herpes Simplex virus 1)
[6] PV1 (Poliovirus 1, Mahoney)
[7] Other viruses

Analysis of RNA/DNA or protein sequences:

[8] Sequence analysis

RNA / protein interactions:

[9] RNA-protein interaction
[10] RNA-RNA interaction
[11] Protein-protein interaction

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Fig. 5 – Software Virus.pro for simulating RNA/DNA virus replication cycles

CoronaVirus.pro - SARS/MERS/2019-nCoV Replication Cycle Simulation

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(1) Attachment and fusion (APN/ACE2/DPP4 receptor)
(2) Endocytosis
(3) Translation of vRNA (ORF1.1)
(4) Assembly of RNA-dependent RNA polymerase (RdRp) and other non-structural proteins (nsp) by proteases
(5) Transcription of subgenomic mRNAs by RdRp
(6) Translation of subgenomic mRNAs and protein synthesis
(7) Assembly into membraneous regions ERGIC
(8) Fusion with plasma membrane and exit

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Fig. 6 – Software Coronavirus.pro for simulating SARS/MERS/2019-nCoV virus replication cycle
Coronavirus.pro includes a preprocessor to convert viral genome sequence file into a plain sequence format (nucleotides list). Preprocessor supports genomes in formats: FASTA, EMBL, GenBank, Stockholm 1.0 and GCG (see fig 8).

3.2 Simulation of translation, protein synthesis and protease cleavage sites

Each subgenomic mRNA transcribed by coronavirus RNA-dependent RNA polymerase (RdRp) is translated into virus proteins by host-cell ribosomes. ORF1.1 and ORF1.2 in SARS-CoV, ORF1.1 to ORF1.4 in MERS-CoV and ORF1.1 to ORF1.6 in 2019-nCoV encode a polypeptide chain of variable length, with a -1 programmed ribosomal frameshift (PRF). This frameshift is followed by a pseudoknot structure located few nucleotides downstream and is able to change reading frame -1 position backward, translating an alternative polypeptide (pp1ab with frameshift). Frameshifting probability is approximately between 5-10%. In same cases, translation starts after a leaky scanning (shunting) [Dinman 2012, Bock et al 2019] or Internal Ribosomal Entry Site (IRES) [Bonnal et al 2003, Mokrejs et al 2006]

After this, viral proteases cleave with enzymatic activity these polypeptides at specific cleavage sites to synthesize non-structural proteins [Kiemer et al 2004]. Protease cleavage sites are predicted depending on protease family (aspartic, cysteine, metallo or serine protease) and through specific cleavage patterns [Song et al. 2012] Coronavirus proteases are a papain-like (PL2pro) and a cysteine 3C-like proteinase (3CLpro) synthesized from nsp3 and nsp5, respectively [Chen et al 2005].
Coronavirus.pro simulates proteolytic effect of coronavirus proteases PL2pro and 3CLpro through multiple protease pattern sequences (table 3). Most of these sequences have been previously validated in some research studies and others have been proposed by comparison with protein databases, as UniProt or NCBI, and recursive simulations with the software [Kiemer et al 2004, Sulea et al 2006, Ramos-Pascual 2019].

| PCS | Sequence | SARS-CoV | MERS-CoV |
|-----|----------|----------|----------|
| [1] | V5QIQ↓SRLT | S1/S2-S0 | - |
| [2] | GKIQQ↓SSLST | S1/S2-S0 | - |
| [3] | GAIQI↓QFTTT | - | S1/S2-S0 |
| [4] | YPKLQ↓ASQAW | M1-M2 | - |
| [5] | SNLQI↓GLEN | N1-N2 | - |
| [6] | ETRVQ↓CSTN | N2-N3 | - |
| [7] | EPRQI↓AVTRX | nsp1-nsp2 | - |
| [8] | LTKLG↓YATQL | nsp2-nsp3 | nsp2-nsp3 |
| [9] | KSSQV↓SVAG | nsp3-nsp3a | - |
| [10] | KNTVQ↓SVQGF | nsp3-nsp3a | - |
| [11] | AQGLQ↓KFYKE | - | nsp3-nsp3a |
| [12] | ETRQI↓CSTN | nsp3a-nsp3b | nsp3a-nsp3b |
| [13] | SLLGQ↓KIVST | nsp3b-nsp4 | - |
| [14] | KVQGQ↓APTWF | nsp3b-nsp4 | - |
| [15] | SAVLQ↓SGFRK | nsp4-nsp5 | nsp4-nsp5 |
| [16] | GSVLQ↓SGVRK | nsp5-nsp6 | - |
| [17] | VATLQ↓SKMSD | nsp6-nsp7 | - |
| [18] | VATLQ↓AENV | nsp7-nsp8a | - |
| [19] | VAAMQ↓SKLTD | nsp8a-nsp8b | nsp6-nsp7 |
| [20] | HSVLQ↓APMST | - | nsp7-nsp8a |
| [21] | AKLQI↓NNEVS | nsp8b-nsp9 | nsp8a-nsp9 |
| [22] | TVQI↓AGNAT | nsp9-nsp10 | nsp9-nsp10 |
| [23] | ELTLMQ↓SADA | nsp10-nsp11/nsp12 | - |
| [24] | ALPOS↓KDNSF | nsp10-nsp11/nsp12 | - |
| [25] | HTVQI↓AVGAC | nsp12-nsp13a | nsp12-nsp13a |
| [26] | VATLQ↓AENV | nsp13a-nsp14 | nsp13a-nsp13b |
| [27] | YKLQI↓QIVTG | nsp13b-nsp14 | - |
| [28] | FTRQI↓SLENV | nsp14-nsp15 | - |
| [29] | TVKQG↓LENS | nsp14-nsp15 | - |
| [30] | YPKLQL↓ASQAW | nsp15-nsp16 | nsp15-nsp16 |

Cleavage sites are identified with a coarse approximation in which each protease cleavage sequence (A) scans through each protein amino acid sequence (B). If ka and kb are respectively the amino acid length of A sequence and protein B, protein is cleaved at positions with the highest Levenshtein distance, calculated as:

```
for (i = 1; i <= ka; i++) d[i][0] = i; for (i = 1; i <= kb; i++) d[0][i] = i;
for (i = 1; i <= ka; i++)
  for (j = 1; j <= kb; j++)
    c = 0;
    if (a[i - 1] == b[j - 1]) { c = 0; }
    else { c = 1; }
    d[i][j] = min(d[i - 1][j] + 1, d[i][j - 1] + 1, d[i - 1][j - 1] + c)
```

4. Results and discussion

4.1 Virus identification: comparison with SARS-CoV and MERS-CoV

Coronavirus.pro has been used with sequence MN908947 (Wuhan seafood market pneumonia virus isolate Wuhan-Hu-1, complete genome) This sequence is specific of the virus type that caused the outbreak in Wuhan of Hubei province (China), the first infection focus (23-Jan-2020), which has been named as 2019-nCoV [NCBI database]

The code has been also applied to other coronavirus types, such as sequences NC004718 (SARS coronavirus, complete genome) and NC019843 (MERS Middle East respiratory syndrome coronavirus, complete genome) [Snijder et al 2003, Moreno et al 2017], which have been taken as reference sequences. Other sequences have been also applied to compare similarity with 2019-nCoV virus genome (see table 4)

| Virus          | Sequence | Date       | Description                                                      | bp    | Comments |
|---------------|----------|------------|------------------------------------------------------------------|-------|----------|
| 2019-nCoV     | MN908947 | 23-JAN-2020| Wuhan seafood market pneumonia virus isolate Wuhan-Hu-1 (2019-nCoV) | 29903 | [ref]    |
| MERS-CoV      | NC019843 | 13-AUG-2018| Middle East respiratory syndrome coronavirus                     | 30119 | [ref]    |
| SARS-CoV      | NC004718 | 13-AUG-2018| SARS-CoV coronavirus                                              | 29751 | [ref]    |
|               | KY417149 | 18-DEC-2017| Bat SARS-like coronavirus isolate Rs4255                        | 29743 | -        |
|               | KY278488 | 01-SEP-2009| SARS coronavirus BJ01 isolate genome sequence                    | 29725 | -        |

4.2 Coronavirus RNA structure

4.2.1 Regulatory regions: 5’utr

Beta-coronaviruses have several stem-loop structures in the 5’utr region (SL1 toSL5C). The first transcription regulating sequence (TRS-L) is placed around the same positions in all beta-coronaviruses (SL3).

MERS-CoV presents a 5’utr region of 356bp, with a TRS-L without SL3. Stem-loop SL4b is only present in 2019-nCoV and MERS-CoV. Furthermore, there are several regulatory sequences (RS) with unknown functionality. Stem-loops SL6 and SL7 are placed into the adjacent ORF1 coding region. [Yang and Leibowitz 2015, Madhugiri et al 2018]

SARS-CoV and 2019-nCoV have a common 5’ utr region of approximately 300bp, with several negative regulatory elements: NRE-I (IL-2R/EBS), NRE-II (Ap1) and NRE-III (Ap1) at positions 101/153/250 and 104/154/251, respectively. Stem-loop SL4b is absent in SARS-CoV, whereas in 2019-nCoV contains a palindrome sequence (-UAAUUA//UAAUUA-) with an unknown function.

Figures 9 to 11 shows 5’utr regions in MERS-CoV, SARS-CoV and 2019-nCoV coronaviruses, obtained through bracket-dot notation from Vienna RNA package and plotted with Varna software.
Fig. 9 – MERS-CoV [1-356bp] - Scheme of the 5’ utr secondary structures (SL1-SL5C)

Fig. 10 – SARS-CoV [1-300bp] - Scheme of the 5’ utr secondary structures (SL1-SL5C)
4.2.2 Regulatory regions: 3’utr

Beta-coronaviruses have a short 3’utr region of approximately 100bp with a stem-loop of variable length followed with a poly(A) tail. This stem-loop, in the case of MERS-CoV is 27bp, including other recognition sequences, different as SARS-CoV and 2019-nCoV coronaviruses, as presented in figure 12. The 3’ utr region of these coronaviruses contains also a conserved pseudo-knot structure of approximately 55bp [Lin et al 1996, Yang and Leibowitz 2015, Peng et al 2016].
4.3 Protease cleavage sites

Coronavirus.pro has predicted all canonical protease cleavage sites in coronavirus polypeptides, from nsp1 to nsp16. Furthermore, the software has identified a protease cleavage site in spike glycoprotein precursors (Sp) in all coronaviruses. This cleavage site splits spike precursors (Sp) into S/S0 proteins, where S0 is the same protease fragment in both precursors.

Another cleavage site has been predicted in membrane protein (M) of 2019-nCoV virus, producing fragments M1/M2. In the case of MERS-CoV nucleocapsid (N), the software has found two protease cleavage sites, N1/N2 and N2/N3. In addition to these cleavage sites, the code has identified other protease cleavage sites giving place to some hypothetical proteins, as nsp3a↓nsp3b, nsp8a↓nsp8b and nsp13a↓nsp13b (see table 5). These cleavage sites must be discussed in detail and supported with other methods.
### Table 5 - Protease cleavage sequences predicted by Coronavirus.pro

| Structural proteins | Protein | PCS | SARS-CoV | PCS | 2019-nCoV | PCS | MERS-CoV |
|---------------------|---------|-----|----------|-----|-----------|-----|----------|
| S1 & S2 S0          | [1.1]   | SQIQE↓SLTT | [1.2]   | GKIQQ↓SLSST | [1.3]   | GAMQ↓GFTTT |       |
| M1 M2               | [2]     | YKLGA↓SQRVG | [2]     | YKLGA↓SQRVA | -       | -         |       |
| N1 N2               | -       | -             | -       | -             | [3]     | NLRQA↓LSEGK |       |
| N2 N3               | -       | -             | -       | -             | [4]     | QRVQG↓STQOR |       |
| nsp1 nsp2           | [5]     | ELNGQ↓AVTRY | [5]     | ELNGQ↓AVTRY | [6]     | DPKKG↓YAQNL |       |
| nsp2 nsp3           | [7]     | RLKGG↓APKIG | [7]     | LKGG↓PITKVT | [7]     | RLKGG↓APKVK |       |
| nsp3 nsp3a          | [8]     | NSVKS↓VAKLCL | [9]     | KNTVK↓SVGGF | [10]    | AGQLK↓KFYKE |       |
| nsp3a nsp3b         | [11/4]  | TRVCE↓TITTN | [11/4]  | TRVEA↓STTVC | [11/4]  | TRVEA↓STTVC |       |
| nsp3b nsp4           | [12]    | SLKGG↓KIVST | [12]    | ALKGG↓KIVNN | [13]    | KIVGG↓APTW |       |
| nsp4 nsp5           | [14]    | SAVLQ↓SGFRK | [14]    | SAVLQ↓SGFRK | [14]    | GVLOQ↓GLVKM |       |
| nsp5 nsp6           | [15]    | GVTFOQ↓GKFKK | [15]    | VTFQG↓AKVRT | [16]    | GVMQG↓SGVKK |       |
| nsp6 nsp7           | [17]    | VATVQ↓SKMDS | [17]    | VATVQ↓SKMDS | [19]    | VAAQQG↓SKLTD |       |
| nsp7 nsp8a          | [18]    | ATLQA↓IASEF | [18]    | ATLQA↓IASEF | [20]    | SVLQA↓TSELK |       |
| nsp8a nsp8b         | [19]    | AAMQR↓KLEKM | [19]    | AAMQR↓KLEKM | [21]    | AVKLQ↓NNEIK |       |
| nsp8b nsp9           | [21]    | AVKLQ↓NNEIK | [21]    | AVKLQ↓NNEIK | [21]    | AVKLQ↓NNEIK |       |
| nsp9 nsp10          | [22]    | TVRLQ↓AGNT | [22]    | TVRLQ↓AGNT | [22]    | TVRLQ↓AGNT |       |
| nsp10 nsp11 & nsp12 | [23]    | EPLMQL↓SADAS | [23]    | PMLQ↓ADAGS | [24]    | ALPQ↓KDSNF |       |
| nsp12 nsp13a        | [25]    | HTVLQ↓AVGAC | [25]    | HTVLQ↓AVGAC | [26/18] | TTLQA↓VSGCV |       |
| nsp13a nsp13b       | [26/18] | VATLQ↓AENVT | [26/18] | VATLQ↓AENVT | [27]    | YKLQ↓QITVG |       |
| nsp14 nsp15         | [28]    | FTRLQ↓SLENV | [28]    | FTRLQ↓SLENV | [29]    | TKVQG↓LENIA |       |
| nsp15 nsp16         | [30/2]  | YPKLQ↓ASSQAW | [30/2]  | YPKLQ↓SSQAW | [30/2]  | TFYPR↓LQSAA |       |

### Non-structural proteins

#### 4.4 Subgenomic mRNA

Most frequent transcription regulating sequence (TRS) in MERS-CoV is TRS2 (5’-acgaac-3’). MERS-CoV transcribes 11 subgenomic mRNAs, with several ORFs translating polypeptide chains (ORF1.1 to ORF1.4) and several fusion proteins [Li et al 2019].

ORF3b is translated with an Internal Ribosome Entry Site (IRES). Other ORF encode some proteins (AP8b, AP7b, AP9b and AP14) with unknown transcription regulating sequences and functionality [Narayanan et al 2008]. ORF8b overlaps nucleocapside gene (N) and encodes an accessory protein AP8b, called AP9b in SARS. Although it is not translated directly by any TRS, some studies have found antibodies specific to this protein in both in vitro and in vivo samples [Sharma et al 2011].

### Tables 6 and 7 present a summary of ORF and proteins identified with Coronavirus.pro in MERS-CoV.

#### Table 6 - MERS-CoV open-reading frames (ORF) and proteins identified with Coronavirus.pro

| mRNA-TRS\(^{(a)}\) | j (bp) | ORF       | proteins (Aa) | Fusion protein (Aa) | Comments |
|---------------------|-------|-----------|---------------|---------------------|----------|
| 1-[2]               | 63    | ORF1.1    | pp1a 4391     | -                   | [nsp1-nsp11] |
|                     |       |           | pp1ab 7078    | -                   | [nsp1-nsp10], [nsp12-nsp16] |
| 2-[1]               | 3904  | ORF1.2    | Ap2 3022      | pp2/nsp3 846       | [nsp3a-nsp11] |
|                     |       |           | pp2ab 5709    | pp2/nsp3 846       | [nsp3a-nsp10], [nsp12-nsp16] |
| 3-[1]               | 11815 | ORF1.3    | pp3a 487      | pp3/nsp7 24        | [nsp8-nsp11] |
|                     |       |           | pp3ab 3174    | pp3/nsp7 24        | [nsp8-nsp10], [nsp12-nsp16] |
| 4-[2]               | 12751 | ORF1.4    | pp4a 226      | pp4/nsp9 72        | [nsp10-nsp11] |
|                     |       |           | pp4ab 2913    | pp4/nsp9 72        | [nsp10-nsp16] |
| 5-[2]               | 21405 | ORF2      | Sp 1354       | -                   | Surface glycoprotein spike precursor |
|                     |       |           | S 1010        | -                   | Surface glycoprotein spike |
|                     |       |           | S0 344        | -                   | S0 glycoprotein |
| 6-[2]               | 25521 | ORF3      | Ap3 103       | -                   | Accessory protein Ap3 |
| 7-[1]               | 25843 | ORF4a     | Ap4a 109      | -                   | Accessory protein Ap4a |
| 8-[2]               | 25928 | ORF4b     | Ap4b 246      | -                   | Accessory protein Ap4b |
| 9-[2]               | 26833 | ORF5      | Ap5 224       | -                   | Accessory protein Ap5 |
| 10-[2]              | 27583 | ORF6      | E 82          | -                   | Envelope protein |
| 11-[2]              | 27838 | ORF7      | M 219         | -                   | Membrane protein |
|                     |       |           | ORF8a N 413   | Nucloecapsid phosphoprotein |
|                     |       |           | N1/N2/N3 223/166/25 | - | N1/N2/N3 protease fragments |

\(^{(a)}\)Transcription regulating sequence: [1] TRS1 - 5’-cuaaac-3’ // [2] TRS2 - 5’-acgaac-3’
### Table 7 - MERS-CoV open-reading frames (ORF) with unknown TRS predicted by Coronavirus.pro

| mRNA | j (bp) | ORF   | proteins (Aa) | Comments                        |
|------|--------|-------|---------------|--------------------------------|
| a    | >25532 | ORF3b | AP3b 66       | Accessory protein AP3b, IRES translation |
| b    | >28570 | ORF8b | AP8b 113/105/90 | Accessory proteins AP8b1-b7 |
| c    | >28990 | ORF14 | AP14a/b 42/108 | Accessory proteins AP14a/b |

SARS-CoV includes a transcription regulating sequence TRS which is not in MERS virus type, TRS3 (5’-cuaaacgaac-3’), also present in 2019-nCoV. SARS-CoV has several accessory protein (AP3a, AP6, AP7a), which are translated directly from mRNAs. In the case of AP6, although in some studies is named as nsp6, it is not processed by any protease, as other non-structural proteins. Other proteins, as AP11 is only characteristic from SARS-CoV. Furthermore, the shortest mRNA (ORF15), with a length of 263bp, translates no significant proteins and has an unknown functionality. Tables 8 and 9 show open-reading frames (ORF) and proteins (structural, non-structural, accessory and fusion) for SARS identified with Coronavirus.pro.

### Table 8 - SARS-CoV open-reading frames (ORF) and proteins identified with Coronavirus.pro

| mRNA-TRS(a) | j (bp) | ORF | proteins (Aa) | Fusion protein (Aa) | Comments                  |
|-------------|--------|-----|---------------|---------------------|--------------------------|
| 1-[3]       | 63     | ORF1.1 | pp1a 4383     | -                   | [nsp1-nsp11]             |
|             |        |      | pp1ab 7074    | -                   | [nsp1-nsp10], [nsp12-nsp16] |
| 2-[1]       | 3665   | ORF1.2 | AP2 50        | -                   | unknown                  |
|             |        |      | pp2a 3095     | pp2/nsp3 894        | [nsp4-nsp11]             |
|             |        |      | pp2ab 5786    | pp2/nsp3 894        | [nsp4-nsp10], [nsp12-nsp16] |
|             |        |      | pp2b 2628     | pp3/nsp12 856       | [nsp13-nsp16]            |
| 3-[1]       | 3800   | ORF1.2 | AP2 50        | -                   | unknown                  |
|             |        |      | pp2a 3095     | pp2/nsp3 894        | [nsp4-nsp11]             |
|             |        |      | pp2ab 5786    | pp2/nsp3 894        | [nsp4-nsp10], [nsp12-nsp16] |
|             |        |      | pp2b 2628     | pp3/nsp12 856       | [nsp13-nsp16]            |
| 4-[3]       | 21482  | ORF2b | S1p 1255      | -                   | Surface glycoprotein Spike precursor |
|             |        |      | S1 917        | -                   | Surface glycoprotein Spike |
|             |        |      | S0 339        | -                   | S0 protease fragment    |
| 5-[1]       | 21913  | ORF2a | S2p 1112      | -                   | Surface glycoprotein Spike precursor |
|             |        |      | S2 774        | -                   | Surface glycoprotein Spike |
|             |        |      | S0 339        | -                   | S0 protease fragment    |
| 6-[2]       | 25260  | ORF3a | AP3a 274      | -                   | Accessory protein AP3a (SARS acsp3) |
| 7-[2]       | 26109  | ORF4  | E 76         | -                   | Envelope protein        |
| 8-[3]       | 26344  | ORF5  | M 221        | -                   | Membrane protein        |
| 9-[2]       | 26913  | ORF6  | AP6 63       | -                   | Accessory protein AP6 (SARS nsp6) |
| 10-[2]      | 27267  | ORF7a | AP7a 122     | -                   | Accessory protein AP7a  |
| 11-[3]      | 27769  | ORF8a | AP8a 40      | -                   | Accessory protein AP8a  |
| 12-[2]      | 28106  | ORF9a | N 422        | -                   | Nucleocapsid phosphoprotein (p9a) |
| 13-[1]      | 29489  | ORF15 | -            | -                   | -                        |

(a) Transcription regulating sequence: [1] TRS-1 - 5'-cuaaac-3' // [2] TRS2 - 5'-acgaac-3' // [3] TRS3 - 5'-cuaaacgaac-3'

### Table 9 - SARS-CoV open-reading frames (ORF) with unknown TRS predicted by Coronavirus.pro

| mRNA-TRS(a) | j (bp) | ORF | proteins (Aa) | Comments                        |
|-------------|--------|-----|---------------|--------------------------------|
| a           | >25478 | ORF3b | AP3b 175      | Accessory protein AP3b, IRES translation |
| b           | >25640 | ORF3b | AP3b 2 142    | Accessory protein AP3b2         |
| c           | >27273 | ORF7b | AP7b 45       | AP7b                           |
| d           | >27779 | ORF8b | AP8b 85       | AP8b                           |
| e           | >28120 | ORF9b | AP9b 98       | Accessory protein AP9b, MA15 ExoN1 |
| f           | >28130 | ORF11 | AP11 73       | AP11                           |
| g           | >28500 | ORF14 | AP14a/a 71/105 | AP14a/b                       |

16
2019-nCoV coronavirus presents relative differences in transcription and subgenomic mRNAs translation with other beta-coronavirus. This virus initiates transcription in 20 TRS sites, transcribing more types of subgenomic mRNAs than SARS/MERS. 2019-nCoV synthesizes around 9 types of fusion proteins, which are remarkably more than SARS/MERS coronavirus, and it is expected than the concentration of non-structural proteins, specially nsp12 (RdRp), nsp3 (PL2pro) and nsp5(3CLpro) is also higher. This fact could be related with the most severe health effects (toxicity) and highest infectivity on host patients than other betacoronavirus.

Furthermore, 2019-nCoV transcribes a mRNA (ORF15), with the shortest length (374bp), with no significant proteins encoded, as also present in SARS-CoV. Finally, 2019-nCoV virus translates another accessory protein, AP12, specific of this virus type.

Table 10 and 11 shows open-reading frames (ORF) and proteins (structural, non-structural, accessory and fusion) identified with Coronavirus.pro software in 2019-nCoV virus.

**Table 10 - 2019-nCoV open-reading frames (ORF) and proteins identified with Coronavirus.pro**

| mRNA-TRS | j (bp) | ORF | proteins (Aa) | Fusion proteins (Aa) | Comments |
|----------|-------|-----|---------------|----------------------|----------|
| 1-[3]    | 66    | ORF1.1 | pp1a 4406     | -                    | [nsp1-nsp11] |
|          |       |       | pp1ab 7097    | -                    |          |
| 2-[1]    | 753   | ORF1.2 | pp2a 4233     | pp2/nsp1 8          | [nsp2-nsp11] |
|          |       |       | pp2ab 6923    | pp2/nsp1 8          | [nsp2-nsp10], [nsp2-nsp16] |
| 3-[1]    | 2358  | ORF1.3 | pp3ab 3676    | pp3/nsp2 89         | [nsp3-nsp11] |
|          |       |       | pp3ab 6366    | pp3/nsp2 89         | [nsp3-nsp10], [nsp12-nsp16] |
| 4-[1]    | 3597  | ORF1.4 | AP2a 47       | -                    |          |
|          |       |       | AP2b 52       | -                    |          |
|          |       |       | pp4a 3095     | pp4/nsp3 893        |          |
|          |       |       | pp4ab 5785    | pp4/nsp3 893        |          |
| 5-[1]    | 6936  | ORF1.5 | pp5a 2153     | pp5/nsp3a 170       | [nsp3b-nsp11] |
|          |       |       | pp5ab 4843    | pp5/nsp3a 170       | [nsp3b-nsp10], [nsp12-nsp16] |
| 6-[1]    | 8655  | ORF1.6 | pp6a 1377     | pp6/nsp4 234        | [nsp5-nsp11] |
|          |       |       | pp6ab 4067    | pp6/nsp4 234        | [nsp5-nsp10], [nsp12-nsp16] |
| 7-[1]    | 13730 | ORF1.7 | pp7 2595      | pp7/nsp12 824       | [nsp13-nsp16] |
| 8-[1]    | 16049 | ORF1.8 | pp8 1807      | pp8/nsp12 34        | [nsp13-nsp16] |
| 9-[1]    | 18452 | ORF1.9 | pp9 1019      | pp9/nsp14 375       | [nsp15-nsp16] |
| 10-[1]   | 20384 | ORF1.10 | pp10 374     | pp10/nsp15 76       | nsp16 |
| 11-[3]   | 21552 | ORF2  | S 1274        | -                    | Surface glycoprotein spike precursor |
|          |       |       | S0 936        | -                    | Surface glycoprotein spike |
|          |       |       | S0 338        | -                    | S0 protease fragment |
| 12-[2]   | 25385 | ORF3a | Ap3a 276      | -                    | (SARS ORF3/ORF3a/X1/U274) |
| 13-[2]   | 26237 | ORF4  | E 76          | -                    | Envelope protein |
| 14-[3]   | 26469 | ORF5  | M 223         | -                    | Transmembrane protein |
|          |       |       | M 183         | -                    | M1 protease fragment |
|          |       |       | M2 40         | -                    | M2 protease fragment |
| 15-[2]   | 27041 | ORF6  | AP6 62        | -                    | (SARS ORF6/p6) |
| 16-[2]   | 27388 | ORF7a | AP7a 122      | -                    | (SARS ORF8/U122/X4/ORF7a) |
| 17-[1]   | 27644 | ORF7b | AP7a 43       | -                    | (SARS ORF7b) |
| 18-[3]   | 27884 | ORF8  | AP8 122       | -                    | (SARS ORF8) |
| 19-[3]   | 28256 | ORF9  | N 420         | -                    | Nucleocapsid phosphoprotein (p9a) |
| 20-[1]   | 29530 | ORF15 | -             | -                    |          |

(a) Transcription regulating sequence: [1] TRS1 - 5’-cuaaac-3’ // [2] TRS2 - 5’-acgaac-3’ // [3] TRS3 - 5’-cuaaacgaac-3’
Table 11 - 2019-nCoV open-reading frames (ORF) with unknown TRS predicted by Coronavirus.pro

| mRNA | j (bp)       | ORF   | proteins (Aa) | Comments |
|------|--------------|-------|---------------|----------|
| a    | >25405       | ORF3b1| AP3b1         | 42       |
|      |              | ORF3b2| AP3b2         | 34       |
| b    | >25457       | ORF3b3| AP3b3         | 58       |
|      |              | ORF3b4| AP3b4         | 152      |
| c    | >28274       | ORF9b | AP9b          | 98       |
| d    | >28305       | ORF11 | AP11          | 73       |
| e    | >28359       | ORF12 | AP12          | 43       |
| f    | >28450       | ORF14 | AP14a/b       | 74/187   |

In general to all of these betacoronaviruses, there are several accessory proteins which expression in vivo and in vitro has not been proved, and therefore its function is still unknown. It is the case of accessory protein AP2 in SARS/MERS and AP2a/b in 2019-nCoV.

4.5 Coronavirus proteins

There are considerable differences between spike glycoproteins. For example, the number of spike glycoproteins is variable with MERS and also between SARS virus types. KY417149 (SARS) virus sequence encodes three spike glycoprotein precursors of different amino acid lengths (S1p, S2p and S3p), which later are processed by virus protease into S1, S2 and S3 spikes, with a common fragment S0. In the case of, NC004718 and AY278488 (SARS), it synthesizes two spike precursors (S1p and S2p), whereas 2019-nCoV and MERS, only one is processed. Spike glycoproteins from the same virus, although having different lengths, are estimated with a 100% identity, as observed from their identity matrices.

In the case of other proteins (N, M and E), it can be observed that this virus is more close related to SARS than to MERS, as also discussed previously. However, it presents also around 10% differences with other SARS, so it could be considered as a different virus type.

All these proteins have been aligned with Clustal 1.2 to compare similarities [Higgins 1994, Brown et al 1998] (see Annex A for alignment details).

Table 12 compares structural proteins in these genome sequences of beta-coronaviruses.
Table 12 - Comparison of structural proteins of SARS-CoV, MERS-CoV and 2019-nCoV

| # | Structural proteins length (Aa) | Spike glycoprotein (S) - Percent Identity Matrix - created by Clustal2.1 |
|---|---------------------------------|---------------------------------------------------------------------|
| 1 | 1: NC019843 (MERS-CoV)          | 100.00 27.98 29.60 72.98 29.60 28.27 27.36 26.91 44.77 |
| 2 | 2: MN908947 (2019-nCoV)         | 72.98 100.00 100.00 99.67 99.74 70.57 75.98 74.19 92.92 |
| 3 | 3: NC004718 (SARS-CoV)          | 99.67 100.00 100.00 99.74 99.74 73.94 78.19 78.19 92.92 |
| 4 | 4: KY417149 (SARS-CoV)          | 99.74 100.00 100.00 100.00 100.00 74.06 78.32 78.32 92.92 |
| 5 | 5: KY417149 (SARS-CoV)          | 99.74 100.00 100.00 100.00 100.00 74.06 78.32 78.32 92.92 |
| # |                                | S0 protein - Percent Identity Matrix - created by Clustal2.1 |
| 1 | 1: NC019843 (MERS-CoV)          | 100.00 41.14 41.74 41.74 42.04 |
| 2 | 2: MN908947 (2019-nCoV)         | 41.14 100.00 94.67 94.67 94.67 |
| 3 | 3: NC004718 (SARS-CoV)          | 41.74 94.67 100.00 100.00 98.22 |
| 4 | 4: KY417149 (SARS-CoV)          | 41.74 94.67 100.00 100.00 98.22 |
| 5 | 5: KY417149 (SARS-CoV)          | 42.04 94.67 98.22 98.22 100.00 |
| # |                                | Nucleocapsid (N) - Percent Identity Matrix - created by Clustal2.1 |
| 1 | 1: NC019843 (MERS-CoV)          | 100.00 48.47 48.09 48.09 48.09 |
| 2 | 2: MN908947 (2019-nCoV)         | 48.47 100.00 89.29 89.52 89.52 |
| 3 | 3: KY417149 (SARS-CoV)          | 48.09 89.29 100.00 99.76 99.76 |
| 4 | 4: NC004718 (SARS-CoV)          | 48.09 89.52 99.76 100.00 100.00 |
| 5 | 5: AY278488 (SARS-CoV)          | 48.09 89.52 99.76 100.00 100.00 |
| # |                                | Membrane (M) - Percent Identity Matrix - created by Clustal2.1 |
| 1 | 1: NC019843 (MERS-CoV)          | 100.00 40.00 42.27 42.73 42.73 |
| 2 | 2: MN908947 (2019-nCoV)         | 40.00 100.00 88.74 89.64 89.64 |
| 3 | 3: KY417149 (SARS-CoV)          | 42.27 88.74 100.00 98.20 98.20 |
| 4 | 4: NC004718 (SARS-CoV)          | 42.73 89.64 98.20 100.00 100.00 |
| 5 | 5: AY278488 (SARS-CoV)          | 42.73 89.64 98.20 100.00 100.00 |
| # |                                | Envelope (E) - Percent Identity Matrix - created by Clustal2.1 |
| 1 | 1: NC019843 (MERS-CoV)          | 100.00 34.67 34.21 34.21 34.21 |
| 2 | 2: MN908947 (2019-nCoV)         | 34.67 100.00 96.05 96.00 96.05 |
| 3 | 3: KY417149 (SARS-CoV)          | 34.21 96.05 100.00 100.00 100.00 |
| 4 | 4: NC004718 (SARS-CoV)          | 34.21 96.00 100.00 100.00 100.00 |
| 5 | 5: AY278488 (SARS-CoV)          | 34.21 96.05 100.00 100.00 100.00 |
| # |                                | Non-structural proteins length (Aa) |
| 1 | 1: NC019843 (MERS) 181 672 1361 | nsp12/nsp3a/nsp3b/nsp3c/nsp4/nsp5/nsp6/nsp7/nsp8a/nsp8b/nsp9/nsp10/nsp11 |
| 2 | 2: MN908947 (2019-nCoV)         | 181 638 1362 218 340 500 306 290 84 198 - 110 141 14 |
| 3 | 3: NC004718 (SARS) 180 639 1385 | |
| 4 | 4: AY278488 (SARS) 180 638 1354 | |
| 5 | 5: KY417149 (SARS) 180 638 1582 | |

| # | nsp12/nsp3a/nsp3b/nsp3c/nsp4/nsp5/nsp6/nsp7/nsp8a/nsp8b/nsp9/nsp10/nsp11 |
| 1 | 1: NC019843 (MERS) 933 236 362 524 340 306 |
| 2 | 2: MN908947 (2019-nCoV) 932 601 - 527 346 299 |
| 3 | 3: NC004718 (SARS) 931 601 - 527 346 299 |
| 4 | 4: AY278488 (SARS) 932 601 - 527 346 299 |
| 5 | 5: KY417149 (SARS) 932 601 - 527 346 299 |
2019-nCoV protein sequences have been compared with SARS/MERS, through a distance estimator, calculated as $id(\%) = (1-d/L)\times100$, where $d$ is the Levenshtein distance between both sequences and $L$ is the protein length of the SARS/MERS reference protein sequence. Although there are other distance estimators (Needleman-Wunsch, Smith-Waterman, Damerau-Levenshtein), the Levenshtein distance is an accurate estimator for high similar sequences.

In the case of MERS, no identity has been found in any protein (< 50%). Table 13 compares 2019-nCoV proteins with several virus genome sequences of SARS-CoV. As observed, most of non-structural proteins (nsp1, nsp3b and nsp5 to nsp16), accessory proteins AP7a/b and structural proteins M, N and E have the highest percents of similarity (>70%), proving that this virus is more close related with SARS type than MERS. Glycoprotein spike (S), most of accessory proteins (except AP7a/b, AP11 and AP14a) and non-structured proteins nsp2 to nsp3a and nsp4 have low similarity (< 50 %), proving that those proteins are characteristics of this virus type, and potential targets for specific vaccines and antiviral drugs. The fact that non-structural proteins are similar to SARS, indicates that antiviral drugs could be effective also to this virus.

Table 13 - Comparison of structural, non-structural and accessory proteins of 2019-nCoV with SARS-CoV

| Protein | NC004718 | KY417149 | AY278488 |
|---------|----------|----------|----------|
|         | % Id$^{(1)}$ | % Id$^{(2)}$ | % Id$^{(3)}$ |
| Structural |         |          |          |
| N       | 90.48    | 90.24    | 90.48    |
| M       | 90.58    | 89.24    | 90.58    |
| M1      | 91.26    | 90.16    | 91.80    |
| M2      | 82.50    | 85.00    | 85.00    |
| E       | 94.74    | 94.74    | 94.74    |
| S       | < 50 %   | < 50 %   | < 50 %   |
| Sp      |          |          |          |
| Non-structural |         |          |          |
| Nsp1    | 83.89    | 85.00    | 84.44    |
| Nsp2    | < 50 %   | < 50 %   | < 50 %   |
| Nsp3    | < 50 %   | < 50 %   | < 50 %   |
| Nsp3a   |          |          |          |
| Nsp3b   | 87.65    | 87.94    | 88.24    |
| Nsp4    | < 50 %   | < 50 %   | < 50 %   |
| Nsp5    | 95.44    | 95.77    | 95.77    |
| Nsp6    | 87.54    | 87.20    | 87.20    |
| Nsp7    | 97.62    | 100      | 98.81    |
| Nsp8a   | 94.64    | 98.21    | 98.21    |
| Nsp8b   | 95.74    | 97.16    | 97.16    |
| Nsp9    | 95.58    | 97.35    | 97.35    |
| Nsp10   | 96.43    | 97.14    | 96.43    |
| Nsp11   | 84.62    | 76.92    | 76.92    |
| Nsp12   | 96.24    | 96.03    | 96.24    |
| Nsp13   | 99.50    | 99.50    | 99.67    |
| Nsp14   | 94.69    | 95.64    | 95.07    |
| Nsp15   | 88.15    | 88.73    | 88.73    |
| Nsp16   | 93.31    | 94.31    | 93.31    |
| Accessory |         |          |          |
| AP2a    | < 50 %   | < 50 %   | < 50 %   |
| AP2b    |          |          |          |
| AP3a    |          |          |          |
| AP3b1   |          |          |          |
| AP3b2   |          |          |          |
| AP3b3   |          |          |          |
| AP3b4   |          |          |          |
| AP6     | 67.74    |          |          |
| AP7a    | 85.25    | 87.70    | 85.25    |
| AP7b    | 79.55    | 81.82    | 79.55    |
| AP8     | < 50 %   | < 50 %   | < 50 %   |
| AP9b    |          |          |          |
| AP11    | 76.71    | 73.97    | 76.71    |
| AP12    | < 50 %   | < 50 %   | < 50 %   |
| AP14a   | 74.32%   | 74.32    | 74.32    |
| AP14b   | < 50 %   | < 50 %   | < 50 %   |
As 3CLpro (nsp5) and RdRp (nsp12) have >90% similarities with SARS, some antiviral drugs, such as protease inhibitors or RNA-dependent RNA polymerase inhibitors could be effective to this virus type. Nevertheless, further comparisons would be required, including other types of estimators.

5. Conclusions

Coronavirus.pro software provides an accurate and reliable simulation model of Coronaviruses replication cycles: SARS/MERS/2019-nCoV. The code simulates transcription of subgenomic mRNAs, translation, protease cleavage, protein synthesis and virus assembly, including all fusion proteins.

As a result of the analysis, 2019-nCoV can be identified as a beta-coronavirus type SARS-CoV virus with high confidence, named SARS-CoV2, and it is consistent with other recent research analysis. Similarities have been found in 5’utr and 3’utr regions, protease cleavage sites and amino acid composition of both structural and non-structural proteins [Ceraolo and Giorgi 2020, Gorbatenya et al. 2020, Wu et al 2020]. However, there are still differences between both coronavirus (SARS-CoV and 2019-nCoV), as the number of spike precursors and accessory proteins.

Coronavirus.pro is able to identify virus type and family, comparing virus genome and proteins with protein and RNA motifs databases. In this case, 2019-nCoV has been identified as a beta-coronavirus SARS in more than 70% than with MERS. However several differences have been found with SARS/MERS. 2019-nCoV has more transcription regulating sequences (TRS) interspaced in the genome and consequently, is producing more subgenomic mRNAs and more fusion proteins during RdRp transcription, which could explain more severe health effects and infectivity than SARS/MERS.

The software has identified those proteins characteristics of 2019-nCoV: Spike S, AP3a, AP3b, AP8, AP9b, AP12 and AP14b and nsp2/3/3a, with similarity < 50 % with other beta-coronaviruses.

Coronavirus.pro has predicted also some accessory proteins in all beta-coronavirus which have not been previously described, called AP2 in SARS-CoV and MERS-CoV, and AP2a/AP2b in 2019-nCoV, respectively. These proteins are encoded in the same genetic region as PL2pro protease (nsp3) and are translated before ORF1.2 (SARS/MERS) and ORF1.4 (2019-nCoV). If they are expressed in vivo or in vitro is not clearly understood, as they could be part of a leaky scanning/shunting mechanism.

The software has predicted some additional protease cleavage sites, giving place to some hypothetical proteins, as nsp3a↓nsp3b, nsp8a↓nsp8b, nsp13a↓nsp13b, M1↓M2 and N1↓N2↓N3. These cleavage sites must be discussed and supported in detail with other methods.

As a conclusion, Coronavirus.pro (2019-nCoV) is able to identify virus genomes and provides in short times useful results (FASTA files of virus proteins and RNA secondary structures). Future research will be focused in interactions between RNA and protein sequences and intracellular processes, fusion protein synthesis, RNA packaging and virus assembly, as carried out before with HIV virus with Monte Carlo simulations. These results will be applied to develop preventive actions (vaccines), diagnostic methods (real time RT-PCR or ELISA tests), and antiviral drugs (fusion inhibitors, RdRp inhibitors or PL2pro/3CLpro protease inhibitors).
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Annex A - Sequence alignment of Spike Glycoprotein

A.1 Spike glycoproteins (S)

Reference sequence (1): NC019843-S
Identities normalized by aligned length.
Colored by: identity

| Rank | Accession | Cov | PID |
|------|-----------|-----|-----|
| 1    | NC019843-S | 100.0% | 100.0% |
| 2    | NC004718-S1 | 88.1%  | 24.0% |
| 3    | NC004718-S2 | 74.3%  | 26.5% |
| 4    | AY278405-S1 | 88.1%  | 24.0% |
| 5    | AY278405-S2 | 74.3%  | 26.5% |
| 6    | MN908947-S  | 89.3%  | 24.5% |
| 7    | KY417149-S1 | 84.0%  | 23.4% |
| 8    | KY417149-S2 | 86.8%  | 22.8% |
| 9    | KY417149-S3 | 23.7%  | 43.5% |
|      | Consensus   | 100%  |    |

| Rank | Accession | Cov | PID |
|------|-----------|-----|-----|
| 1    | NC019843-S | 100.0% | 100.0% |
| 2    | NC004718-S1 | 88.1%  | 24.0% |
| 3    | NC004718-S2 | 74.3%  | 26.5% |
| 4    | AY278405-S1 | 88.1%  | 24.0% |
| 5    | AY278405-S2 | 74.3%  | 26.5% |
| 6    | MN908947-S  | 89.3%  | 24.5% |
| 7    | KY417149-S1 | 84.0%  | 23.4% |
| 8    | KY417149-S2 | 86.8%  | 22.8% |
| 9    | KY417149-S3 | 23.7%  | 43.5% |
|      | Consensus   | 100%  |    |

| Rank | Accession | Cov | PID |
|------|-----------|-----|-----|
| 1    | NC019843-S | 100.0% | 100.0% |
| 2    | NC004718-S1 | 88.1%  | 24.0% |
| 3    | NC004718-S2 | 74.3%  | 26.5% |
| 4    | AY278405-S1 | 88.1%  | 24.0% |
| 5    | AY278405-S2 | 74.3%  | 26.5% |
| 6    | MN908947-S  | 89.3%  | 24.5% |
| 7    | KY417149-S1 | 84.0%  | 23.4% |
| 8    | KY417149-S2 | 86.8%  | 22.8% |
| 9    | KY417149-S3 | 23.7%  | 43.5% |
|      | Consensus   | 100%  |    |

| Rank | Accession | Cov | PID |
|------|-----------|-----|-----|
| 1    | NC019843-S | 100.0% | 100.0% |
| 2    | NC004718-S1 | 88.1%  | 24.0% |
| 3    | NC004718-S2 | 74.3%  | 26.5% |
| 4    | AY278405-S1 | 88.1%  | 24.0% |
| 5    | AY278405-S2 | 74.3%  | 26.5% |
| 6    | MN908947-S  | 89.3%  | 24.5% |
| 7    | KY417149-S1 | 84.0%  | 23.4% |
| 8    | KY417149-S2 | 86.8%  | 22.8% |
| 9    | KY417149-S3 | 23.7%  | 43.5% |
|      | Consensus   | 100%  |    |
A.2 S0 protein from Spike glycoprotein precursor (Sp)
A.3 Envelope protein (E)

Reference sequence (1): NC019843
Identities normalised by aligned length.
Colored by: identity

|    | cov | pid | seq                  |
|----|-----|-----|----------------------|
| 1  | 100.0% 100.0% | NC019843 | MLPGIPQFNPSPDIYVCAFLVQATLWCHPGPAVLRGDAQCHQFGNLITIAAPLYYN |
| 2  | 91.5% 32.9% | MNSGSHTRLNNSVALLFLAFVVLWTLQAIATTLCAYCCHNIVYSSWYSHAYS |
| 3  | 92.7% 32.9% | MKPSGSHTRLNNSVALLFLAFVVLWTLQAIATTLCAYCCHNIVYSSWYSHAYS |
| 4  | 92.7% 32.9% | MNSGSHTRLNNSVALLFLAFVVLWTLQAIATTLCAYCCHNIVYSSWYSHAYS |
| 5  | 92.7% 32.9% | MNSGSHTRLNNSVALLFLAFVVLWTLQAIATTLCAYCCHNIVYSSWYSHAYS |
|    |     |     | consensus/100%       |
|    |     |     | hpsckckshkkcn.hhhhlshhll.lvehahtrbvgc.shshbsh.bckhks.vshvbks |
|    |     |     | consensus/50%        |
|    |     |     | hpsckckshkkcn.hhhhlshhll.lvehahtrbvgc.shshbsh.bckhks.vshvbks |
|    |     |     | consensus/80%        |
|    |     |     | ysvpseyvlsnywllflafvvlwvlqaiattlcaycchnvyswlyvysyshvys |
|    |     |     | consensus/70%        |
|    |     |     | ysvpseyvlsnywllflafvvlwvlqaiattlcaycchnvyswlyvysyshvys |

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A.4 Membrane protein (M)

Reference sequence (1): NC019843
Identities normalized by aligned length.
Colored by: identity

| cov | pid | 1 |
|-----|-----|---|
| 1   | NC019843 | 100.0% 100.0% |
| 2   | MN908947  | 100.0% 39.0% |
| 3   | KY417149  | 100.0% 41.4% |
| 4   | NC006738  | 100.0% 41.9% |
| 5   | AX270488  | 100.0% 41.9% |
|     | consensus/100% |
|     | consensus/90% |
|     | consensus/80% |
|     | consensus/70% |

| cov | pid | 01 |
|-----|-----|---|
| 1   | NC019843 | 100.0% 100.0% |
| 2   | MN908947  | 100.0% 39.0% |
| 3   | KY417149  | 100.0% 41.4% |
| 4   | NC006738  | 100.0% 41.9% |
| 5   | AX270488  | 100.0% 41.9% |
|     | consensus/100% |
|     | consensus/90% |
|     | consensus/80% |
|     | consensus/70% |

| cov | pid | 161 |
|-----|-----|-----|
| 1   | NC019843 | 100.0% 100.0% |
| 2   | MN908947  | 100.0% 39.0% |
| 3   | KY417149  | 100.0% 41.4% |
|     | consensus/100% |
|     | consensus/90% |
|     | consensus/80% |
|     | consensus/70% |
A.5 Nucleocapsid (N)

Reference sequence (1): NC019843
Identities normalized by aligned length.
Colored by: identity

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