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New short RNA motifs potentially relevant in the severe acute respiratory syndrome coronavirus 2 genome

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Abbreviations: NT, nucleotide; tCP, triplet composon; SARS, severe acute respiratory syndrome, COVID-19, coronavirus disease 2019; orf, open reading frame; pp, polyprotein; NSp, non-structural protein

Keywords: COVID-19, SARS-CoV-2, evolution, triplet composon, coronavirus

[†] *In Memoriam:* Prof. Carlos Alonso Bedate.

Abstract

As time passes, identifying new pharmacological targets is becoming more difficult. Shortly, it will be necessary to devise new strategies to tackle the problem. The coronavirus disease outbreak caused by the severe acute respiratory syndrome coronavirus 2, represents a threat to human health serving as example from what we just said. The present study was aimed to collect a set of short RNA motifs with potential biological impact, most of which have not been observed heretofore. Categorizing RNA triplets by their gross-composition, the study collected 88 short RNA motifs, shared by most coronavirus genera independent on the percent identity between genomes. Selected motifs contain all nearest-neighbours of the triplets A, T, G and A, C, G. The high percent identity between severe acute respiratory syndrome coronavirus genomes makes it difficult these peptides to be found by current methods. The results provide 50 motifs in the 1a polyprotein-encoding orf, 27 in the 1b polyprotein-encoding orf, 5 in the spike-encoding orf and 6 in the nucleocapsid-encoding orf. They also provide insights about the validity of the procedure, confirming some motifs interspersed or attached to known relevant functional fragments of the genome, although most of them have not yet been associated to any known function. The high level of preservation of these motifs in most coronavirus genera suggest they might have potential to be used for diagnostic, in vaccines, or as substrate for protease inhibitors.

Introduction

Coronaviridae family contains enveloped, positive-sense, single-stranded RNA viruses. The coronavirus (CoV) taxonomy, by the Coronavirus Study Group of the International Committee for Taxonomy of Viruses (ICTV), classify CoVs into four genera: alpha, beta, gamma and delta¹⁻³. Moreover, bat CoVs are likely the gene source of alpha and beta genera, and avian CoVs are likely the gene source of gamma and delta genera³⁻⁵.

In recent years there have been three zoonotic outbreaks of beta-CoVs: the severe acute respiratory syndrome 1 (SARS-CoV-1); the Middle East respiratory syndrome (MERS-CoV) and the severe acute respiratory syndrome 2 (SARS-CoV-2)⁶. The spread of the pandemic caused by the beta-SARS-CoV-2 is affecting all around the world^{7,8}. After beta-SARS-CoV-2 genome isolate⁹, a study showed that genomes from both bats and pangolin species would be natural reservoirs of the SARS-CoV-2, highlighting the high identity between genomes of these species and the human SARS-CoV-2¹⁰. The high genome identity observed when different beta-SARS-CoV genomes are compared, make it difficult to outline a strategy for distinguish highly conserved short RNA motifs with possible biological interest. Consequently, an approximation to the issue independently on the percent identity between genomes would be desirable.

Categorizing NT triplets by their gross-composition, resulted to be a useful strategy when applied to issues regarding coevolution or gene clustering^{11,12}; an analysis between distant strains of picornavirus genomes, as of the foot-and-mouth disease virus and of the human rhinovirus, gave as result, some NT sequence correlations not observed heretofore¹³. Briefly, the strategy proposed works as follows: i) the genome is read fully-overlapping, taking into account the context of each NT of the genome. ii) each NT triplet is categorized by their gross composition; such categorizations, called triplet composons (or tCPs), generate a tCP sequence that can be compared with other tCP sequences. Comparing tCP sequences let the access to short RNA motifs tCP conserved; Short motifs are hard to be found mainly when the percent identity among genomes is very high. Short RNA motifs, here in collected, are clearly distinguished from the known short linear motifs or SLiMs¹⁴ that are peptides mediating protein-protein interactions

having a functional interaction interface encoded in a short and poorly conserved sequence¹⁵. The goal of the present study was to provide proof-of-concept for a rational approach to obtain short highly conserved RNA motifs potentially relevant.

Methods

Data availability. The CoV genomes were obtained from the GenBank®, <https://www.ncbi.nlm.nih.gov/>¹⁶. Genomes set to be studied would be: human coronavirus (HCoV)¹⁷, porcine epidemic diarrhoea virus (PEDV)¹⁸, human severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)¹⁹, human severe acute respiratory syndrome coronavirus (SARS-CoV-1)²⁰, civet severe acute respiratory syndrome coronavirus (civet SARS-CoV)²¹, mouse hepatitis virus (MHV)²², bat severe acute respiratory syndrome coronavirus (bat SARS-CoV)²³, avian infectious bronchitis virus (IBV)²⁴ and Common moorhen coronavirus³. CoVs data as the strain, the genus, the accession number and references are in Table 1. Abbreviations are taken from the International Committee on Taxonomy of Viruses (ICTV) <https://talk.ictvonline.org/>. In the paper, T is listed as a nucleotide. Since the viral genomes are RNA, U should be substituted for T. However, very often, RNA is reverse-transcribed into DNA first, and then the DNA is sequenced. This is the reason why GenBank present the single-stranded RNA viral genomes in this way. Moreover, NCBI support indicates that replacing U with T is a GenBank convention, which saves computational resources.

<i>Virus</i>	<i>Strain</i>	<i>Genus</i>	<i>Accession</i>	<i>Length (bp)</i>	<i>references</i>
<i>Human HCoV</i>	229E	Alpha-CoV	AF304460	27317	17
<i>Porcine PEDV</i>	CV777	Alpha-CoV	AF353511	28033	18
<i>Human SARS-CoV-2</i>	WHU01	Beta-CoV	MN988668	29881	19
<i>Human SARS-CoV-1</i>	Urbani	Beta-CoV	AY278741	29727	20
<i>Civet SARS-CoV</i>	SZ3	Beta-CoV	AY304486	29741	21
<i>Murine MHV</i>	A59	Beta-CoV	AY700211	31335	22
<i>Bat SARS-CoV</i>	279/2005	Beta-CoV	DQ648857	29741	23
<i>Avian IBV</i>	Mass 41	Gamma-CoV	AY851295	27475	24
<i>Common-moorhen CoV</i>	HKU21-8295	Delta-CoV	JQ065049	26223	3

Table 1. Genomes of different CoV genera

Numerical analysis. Similarities and dissimilarities among genomes of different CoV genera were analysed by the tCP-method¹². The justification of the method is based in exclusionary multiplet categorizations characterized by the presence or absence of particular bases. 14 different categorizations, or tCPs, were identified (Table 2), each one containing one or six NT triplets depending on whether they are non-degenerated or degenerated¹¹. The RNA was read fully-overlapping avoiding information loss and guaranteeing all triplets are considered in the study, taking into account, thus, the context of each NT in the RNA sequence.

COMPOSON	RNA-TRIPLETS ASSOCIATED TO COMPOSONS
<A>	AAA
<T>	TTT
<G>	GGG
<C>	CCC
<AC>	AAC, CAA, ACA, CCA, ACC, CAC
<AT>	AAT, TAA, ATA, TTA, ATT, TAT
<AG>	AAG, GAA, AGA, GGA, AGG, GAG
<CG>	CCG, GCC, CGC, GGC, CGG, GCG
<GT>	GGT, TGG, GTG, TTG, GTT, TGT
<CT>	CCT, TCC, CTC, TTC, CTT, TCT
<AGC>	AGC, GCA, CAG, ACG, CGA, GAC
<AGT>	AGT, GTA, TAG, ATG, TGA, GAT
<ACT>	ACT, CTA, TAC, ATC, TCA, CAT
<TCG>	TCG, CGT, GTC, TGC, GCT, CTG

Table 2. List of tCPs and their associated NTs

The tCP distribution in CoV genomes is represented in cumulative tCP usage frequency graphs. The cumulative frequency is the sum of all previous tCP appearances along the sequence up to the current length. To simplify, the cumulative graph and its regression line, hereafter called ‘tCP profile’, were projected on the axis representing the genome length by subtracting the cumulative tCP usage frequency from the regression line¹². The profile represents the distribution of differences between the tCP events observed, tCP_o , and the tCP events estimated, tCP_e . It will consider that two RNA sequences share a similar tCP profile when the Pearson correlation coefficient, r , is equal or higher than an arbitrarily cut-off. The election of the cut-off would be done on the basis of the p-value (significance level). It will consider for comparison, cut-offs for $0,7 \leq r < 0,8$ and $r \geq 0,8$. The Pearson correlation coefficient obtained are highly significant for both cut-offs due to the dependence of r on the sample size; in the analysis, the sample size is $>2 \times 10^4$ points. For both cut-offs the r significance was $p < 0,001$. Graphs and statistics were carried out with the package OriginPro 8 SRO V8.0724 (B724) ©OriginLab Corporation.

For assessing the applicability of the method, certain criteria must be fulfilled. i) The tCP identity near 100% was discarded when compared CoV genomes, because impedes looking for SLiMs. Thus, the number of shared tCPs must be significantly lower than 14. ii) Viral genomes must be orthologs, because correlation does not imply causation²⁵ unless we are dealing with highly correlated orthologs²⁶. iii) Last but not least, we must search for distant ortholog genomes to look for RNA SLiMs conserved in most CoV genera. This criterion will be met when comparing beta-SARS-CoVs with genomes of the remaining CoV genera.

Identification of tCPs along CoVs genomes. Identification of tCPs along genomes was done as follows: i) we translated CoV genomes to tCP sequences with the help of Table 2; ii) we compared tCP sequences using the dynamic algorithm created for the global alignment of two sequences²⁷; iii) to compare the tCP profiles of different CoVs we computing the correlation coefficient between them; iv) if the correlation coefficient of a pair of given tCP profiles is higher than the cut-off considered, the corresponding shared tCPs were annotated.

Results

Alignment of full-length CoV genomes. We started comparing genomes of different CoV genera to verify whether they fulfil the requirements to apply the tCP-method (Table 3). The beta-SARS-CoV-2 genome share about 79% NT identity with others beta-SARS-CoV genomes, and <51% with others CoV genera, suggesting that beta-SARS-CoVs may have diverged from other CoV genera. When alpha, gamma, and delta-CoVs were compared it was observed <60% NT identity. The genomes of Civet SARS-CoV and human SARS-CoV-1, however, share almost 100% NT and tCP identities supporting the zoonotic nature of human SARS-CoV-1; they differ in a 29-NT sequence retained by the civet isolate not found in the human isolate²¹, sharing, therefore, all the 14 tCPs. In the other cases, the tCP identity is significantly lower than the NT identity.

To sum up, the results presented revealed that selected CoV genomes fulfil the requirements described in Methods and look like adequate for the application of the tCP method to comparisons with the SARS-CoV-2 genome.

Comparison of tCP profiles among human beta-SARS-CoVs-1 and 2. Beta-SARS-CoV-2 and beta-SARS-CoV-1 genomes were compared to illustrate what and how many tCPs are shared by both. The results showed (Table 4) that six tCPs, <AG>, <CG>, <GT>, <AGT>, <C> and <T>, were shared by both, for $r \geq 0,8$ ($p < 0,001$); high resemblances were observed for these profiles (Figure 1). However, significant dissimilarities were observed among both genomes when the correlation coefficient decreases (Figure 1, profiles <AT> and <G>). The complete panel representing all the 14 tCP profiles from the comparison (Supporting Figure 1) shows, for $r < 0,7$, notable dissimilarities in the remaining profiles. Often, short highly similar fragments are observed interspersed in low similarity regions. Such short fragments would be of interest in local genome studies but they were not considered in this paper.

CoV	HSARS CoV-2	HSARS CoV-1	Civet SARS CoV	Bat SARS CoV	HCoV	PEDV	MHV	IBV	Common moorhen CoV
HSARS CoV-2	100 0	79,2 4,3	79,2 4,4	78,9 4,4	49,5 36,0	48,8 36,6	50,3 34,1	49,7 35,2	47,8 38,5
HSARS CoV-1	<i>57,6</i> 8,7	100 0	99,7 0,1	87,8 2,0	49,4 35,5	48,7 36,6	49,4 35,5	49,7 35,6	47,0 39,0
Civet SARS CoV	<i>57,6</i> 8,7	<i>99,3</i> 0,1	100 0	87,7 2,2	49,5 35,3	48,7 36,6	49,6 35,1	49,6 35,5	47,1 39,0
Bat SARS CoV	<i>59,9</i> 9,1	<i>73,0</i> 3,7	<i>73,0</i> 3,7	100 0	49,3 35,5	48,8 36,6	49,3 35,6	48,8 36,5	47,0 39,1
HCoV	<i>30,0</i> 37,3	<i>29,2</i> 37,7	<i>29,2</i> 37,9	<i>29,3</i> 38,6	100 0	59,3 22,5	48,0 37,7	50,1 34,9	49,5 36,3
PEDV	<i>29,5</i> 38,3	<i>29,6</i> 38,7	<i>29,5</i> 38,5	<i>29,0</i> 38,6	<i>35,8</i> 28,2	100 0	48,2 37,9	49,3 35,9	48,6 36,9
MHV	<i>30,1</i> 36,7	<i>29,5</i> 37,6	<i>29,6</i> 37,5	<i>29,4</i> 37,9	<i>28,8</i> 40,1	<i>29,0</i> 38,9	100 0	48,4 37,4	45,4 41,9
IBV	<i>29,9</i> 36,8	<i>29,2</i> 37,5	<i>30,3</i> 37,1	<i>28,9</i> 38,3	<i>30,3</i> 37,1	<i>29,3</i> 39,0	<i>29,2</i> 38,2	100 0	49,9 35,7
Common moorhen CoV	<i>28,8</i> 38,5	<i>27,9</i> 40,3	<i>28,0</i> 40,4	<i>27,8</i> 40,3	<i>29,7</i> 37,9	<i>29,0</i> 39,9	<i>27,7</i> 42,4	<i>30,4</i> 36,4	100 0

Upper number in the box: **bold**, NT percent identity; *italics*, tCP percent identity. Lower number in the box: percentage of GAPS

Table 3. NT and tCP percent identity between alignments of CoV genomes

Figure 1. tCP profiles when genomes of human beta-SARS-CoV-1 (red) and human beta-SARS-CoV-2 (blue) were compared. For $r \geq 0,8$, tCP profiles <AG>, <CG>, <GT>, <AGT>, <C> and <T> were represented. tCP profiles <AT> and <G> were represented as an example of non-shared tCPs, for $r < 0,7$. For each tCP, Pearson correlation coefficients, r , were annotated.

The shared tCPs for cut-offs $r \geq 0,8$ (black) and $0,7 \leq r < 0,8$ (red) were illustrated in Table 4. Most beta-SARS-CoVs would show a high percent identity ranging in between 78,9% to 99,7% for NTs, and 57,6% to 99,3% for tCPs (Table 3); all comparisons show there are many conserved tCPs (6 to 12). All beta-SARS-CoVs, for $r \geq 0,8$, share the tCPs <T>, <AG>, <GT> and <AGT> and for $0,7 \leq r < 0,8$ share the tCP <AGC>.

Figure 2. Similarities and dissimilarities in the profiles of <AGT> and <G>, when the beta-SARS-CoV-2 genome (red) was compared with genomes from A) alpha-HCoV, B) alpha-PED, C) delta-common moorhen CoV and D) gamma-IBV (blue). tCPs were represented in bold together with the value of the correlation coefficient, r .

Table 4. Shared tCPs in alignments of CoV genomes

<i>tCP alignments</i>	<i>SARS CoV-2</i>	<i>SARS CoV-1</i>	<i>Civet SARS CoV</i>	<i>Bat CoV</i>	<i>HCoV</i>	<i>PEDV</i>	<i>MHV</i>	<i>IBV</i>	<i>Common moorhen CoV</i>
<i>SARS CoV-2</i>	all the 14 tCPs	<C>, <T>, <AG>, <CG>, <GT>, <AGT>, <AC>, <CT>, <ACT>, <AGC>	<C>, <T>, <AG>, <CG>, <GT>, <AGT>, <ACT>, <AC>, <CT>, <AGC>	<G>, <T>, <AG>, <GT>, <AGC>, <AGT>, <CG>, <AT>, <AC>	<G>, <AGC>	<AGT>, <C>, <AGC>, <AT>	<C>	<AGT> <AGC>	<AGT>
<i>SARS CoV-1</i>		all the 14 tCPs	all	<C>, <T>, <A>, <AG>, <GT>, <CT>, <AGC>, <AGT>, <TCG>, <ACT>	<CG>	<C>, <AT>	<AT>, <T>	<AGT>	<AGT>
<i>Civet SAR CoV</i>			all the 14 tCPs	<C>, <T>, <A>, <AG>, <GT>, <CT>, <AGC>, <AGT>, <TCG>	<AGT>, <CG>	<C>, <AT>, <AC>	<AT>, <A>, <GT>	<AC>, <CT>, <AG>, <AGC>	<T>, <C>, <AT>
<i>Bat CoV</i>				all the 14 tCPs	-	-	<GT>, <ACT>, <AGT>	<AC>, <AGC>	<AGT>, <T>
<i>HCoV</i>					all the 14 tCPs	<AG>, <GT>, <AGC>, <AGT>, <ACT>, <AC>, <TCG>	<ACT>	<AC>, <CT>, <AG>, <AGC>	<ACT>, <GT>, <CT>
<i>PEDV</i>						all the 14 tCPs	-	<AC>, <AT>, <AG>	<C>, <ACT>, <AT>
<i>MHV</i>							all the 14 tCPs	-	-
<i>IBV</i>								all the 14 tCPs	<AGT>, <GT>, <AT>, <CT>
<i>Common moorhen CoV</i>									all the 14 tCPs

Black text, tCPs conserved for $r \geq 0,8$; **red text**, tCPs conserved for $0,7 \leq r < 0,8$ ($p < 0,001$; points per profile $n > 2 \times 10^4$). As expected, all the 14 tCPs were conserved in identical genomes.

Comparison of tCP profiles among beta-SARS-CoV-2 and other CoV genera. NT and tCP percent identities falls up to 33% and 46%, respectively, when the beta-SARS-CoV-2 genome was compared with alpha-HCoV, alpha-PEDV, gamma-IBV and delta-common-moorhen genomes (Table 3); results indicate that <AGT> is the only tCP conserved, except for the alpha HCoV genome that conserves the tCP <G> (Table 4). But, what happen with the tCP <G> in HCoV?

Figure 3. Similarities and dissimilarities observed in the <AGC> profile when the beta-SARS-CoV-2 genome (red) was compared A) with genomes from beta-SARS-CoVs as Human SARS-CoV-1, Civet SARS-CoV and bat SARS-CoV; B) with genomes from other CoV genera as alpha-HCoV, alpha-PEDV, beta-MHV, gamma-IBV and delta-common moorhen-CoV. The Pearson correlation coefficient, r , was also represented.

To answer the question, we further investigated the resemblance of both <AGT> and <G> profiles when the genome of SARS-CoV-2 was compared with other alpha-CoVs, as well as, with genomes from other CoV genera. Figure 2 shows profiles of both tCPs obtained from alignments of the SARS-CoV-2 genome with genomes of HCoV, PEDV, IBV and common moorhen-CoV genera. The figure illustrates the changes observed in both tCPs along genomes. <AGT> shows an increase in the correlation coefficient from $r=0,63$ (for HCoV) to $r=0,92$ (for IBV) in different CoV genera. All <AGT> profiles maintain a notable resemblance with the exception of the HCoV 5' region that shows important dissimilarities, explaining the decrease in the correlation coefficient observed in HCoV relative to other CoVs. However, a high identity was observed in the HCoV 3' region of the genome, suggesting that dissimilarity observed in the 5' region of the genome was species specific. <AGT> is shared also by SARS-CoVs suggesting that <AGT> would be evolutionary important in all CoV genera. Data further supporting the notion that even for the case of HCoV the resemblance of tCP profiles is notable despite the local dissimilarity in the 5' region.

Changes most pronounced were observed, however, for <G>. The correlation coefficient relative to SARS-CoV-2 for <G> profile changes notably (Figure 2), increasing progressively from avian ($r=-0,24$) to mammals ($r=0,8$). The profile resemblance observed for <AGT> is, however, lost for <G>, indicating a notable change in the <G> usage between avian and mammals. As it is shown in Table 3, the tCP <AGC> would also be conserved in the most CoV genomes

analysed for a cut-off $r \geq 0,7$ ($p < 0,001$). The high significance of the correlation coefficient ensure that at least 49% of the variance will be shared when CoV genomes are compared (determination coefficient, $r^2 = 0,49$). Therefore, the resemblances of <AGC> profile would be also guaranteed providing useful evolutionary information.

Figure 3 shows, a detailed analysis of <AGC> distribution when the beta-SARS-CoV-2 genome was compared with others beta-SARS-CoVs genomes (Figure 3A) and also with genomes from other CoV genera (Figure 3B). The <AGC> profile shows, in all CoV genera, a correlation coefficient $r > 0,7$ ($p < 0,001$) with the exceptions of both MHV ($r = 0,33$) and common moorhen CoV ($r = 0,47$), although, in both genomes, resemblances in the 5' region were notable (Figure 3B); All profiles maintain, indeed, a substantial resemblance, as expected, including MHV and common moorhen CoV, but in these cases with notable dissimilarities 3' regions of tCP profiles. There are not significant differences in correlation coefficients ($r \approx 0,7$) for <AGC> profile except for bat SARS-CoV ($r = 0,88$), indicating evolutionary closeness between human SARS-CoV-2 and bat SARS-CoV. The tCP <AGC> would be, then, considered as significantly conserved in most CoV genera. Not only it is shared in all SARS-CoVs but also in the most CoV genera.

To sum up, the results presented revealed that two tCPs <AGT> and <ACG> were conserved in nearly all CoV genera. Despite dissimilarities in the HCoV 3' region of the genome and the high similarity in the 5' region for <AGT>, this is in keeping with the idea of a common origin for all CoV genera. We suggest, with a high confidence level, that RNA short motifs generated by tCPs <AGT> and <AGC> would be shared by most CoVs, independently on the NT similarity between their genomes.

List of short RNA motifs tCP conserved in CoV genomes. Having established, with high confidence level, that tCPs <AGT> and <AGC> were conserved by most CoV genera, and that <T>, <AG>, <GT>, <AGT> and <AGC> were conserved in beta-SARS-CoVs we are now interested in identifying short RNA motifs tCP conserved by most CoV genera.

First of all, we will create a target selection strategy to isolate short genomic motifs following the next criteria: i) RNA motifs must be tCP conserved in all beta-SARS-CoVs; the criterion imply that selected motifs will share NT-triplets from <T>, <AG>, <GT>, <AGT> and <AGC>. ii) Selected motifs will contain more than 50% of NT-triplets from the conserved tCPs <AGT> and <AGC>. iii) The RNA fragments chosen would be as long as possible; limited exceptions to this rule would be fragments of shorter length having very high percentages of NTs contained in <AGT> and <AGC>. Following these criteria, Table 5 shows 88 selected RNA motifs, as well as, their associated amino acids. The size, position and percentage of conserved NTs in tCPs <AGT> and <AGC> were also annotated. The SARS-CoV-2 harbours a wide-range of selected RNA motifs ranging in size from 12 to 49 bps (from 4 to 16 amino acids).

From now on, in the main text, we will referred to selected RNA motifs using the notation from the end of Table 5: upper case letters would represent NTs-tCP conserved in all beta-SARS-CoVs; underlined-bold upper case letters represent NTs-tCP conserved in most CoV genera; codons would be represented by separated NT triplets in the correct reading frame and amino acids tCP conserved in most CoV genera would be represented by bold upper case letters below their corresponding codons.

Conserved RNA motifs**	N°	Protein encoding ORF	N° amino acids	Genome location	%
GGTGT <u>GTACCGAAAGGTAAG</u> <u>ATG*</u> GAG <u>AGC</u> CTT GT 5' NC region M E S L	1	pp1a	4	246-278	51
<u>AT</u> GTT <u>ATG</u> GTT <u>GAG</u> CTG <u>GTA</u> GCA GAA V M V E L V A E	2	pp1a	8	512-537	69
<u>T</u> AGT TAC GGC GCC <u>GAT</u> S Y G A D	3	pp1a	5	666-681	69
AAG CTT <u>GAT</u> GGC TTT <u>ATG</u> GGT <u>AGA</u> A K L D G F M G R	4	pp1a	8	1137-1162	60
<u>T</u> GAT TGG CTT <u>GAA</u> GAG <u>AGT</u> D W L E E K	5	pp1a	7	2148-2167	53
AA <u>GCC</u> <u>TTG</u> AAT <u>TTA</u> <u>GGT</u> <u>GAA</u> A L N L G E	6	pp1a	6	2366-2385	60
AAG GGA <u>TTG</u> TAC AGA <u>AAG</u> TGT GTT K G L Y R K C V	7	pp1a	8	2404-2427	50
<u>GC</u> ATT <u>GAT</u> <u>TTA</u> <u>GAT</u> <u>GAG</u> <u>TGG</u> <u>AGT</u> <u>ATG</u> <u>GC</u> I D L D E W S M	8	pp1a	8	2945-2972	79
<u>CA</u> GAT <u>GAG</u> <u>GAT</u> <u>GAA</u> GAA GAA GGT <u>GAT</u> TGT <u>GAA</u> GAA GAA D E D E E E G D C E E E <u>GAG</u> TTT <u>GAG</u> CC E F E	9	pp1a	15	3044-3092	55
<u>AT</u> <u>GAG</u> TAT <u>GGTA</u> E Y G	10	pp1a	3	3104-3115	100
<u>T</u> GAA GAA <u>GAG</u> CAA GAA GAA <u>GAT</u> TGG <u>TTA</u> <u>GAT</u> <u>GAT</u> <u>GAT</u> <u>AGT</u> E E E Q E E D W L D D D S	11	pp1a	13	3177-3216	60
<u>TT</u> GAA <u>GTG</u> AAT <u>AGT</u> TTT <u>AGT</u> GGT T E V N S F S G	12	pp1a	7	3332-3355	67
<u>AGC</u> TTT TTG GAA <u>ATG</u> AAG <u>AGT</u> G S F L E M K S	13	pp1a	7	3828-3850	51
<u>TA</u> GCG AAA GCT <u>TTG</u> AGA AAA <u>GTG</u> CC A K A L R K V	14	pp1a	7	4202-4226	60
T <u>GTA</u> GAG GAG <u>GCA</u> AAG <u>ACA</u> <u>GTG</u> V E E A K T V	15	pp1a	7	4278-4299	59
<u>TA</u> GAT GGT <u>GAA</u> GTT D G E V	16	pp1a	4	4901-4914	86
T <u>TTG</u> AGA GAA <u>GTG</u> AGG <u>AC</u> L R E V R	17	pp1a	5	4947-4964	61
<u>A</u> CGT GTT <u>GAG</u> GCT TTT <u>GAG</u> TA R V E A F E	18	pp1a	6	5145-5165	57
AAG <u>ACA</u> <u>GTA</u> GGT <u>GAG</u> TTA GGT <u>GAT</u> <u>GTT</u> <u>AGA</u> GAA K T V G E L G D V R E	19	pp1a	11	5422-5454	73
AAG GGT <u>GTA</u> GAA GCT GTT <u>ATG</u> TAC <u>ATG</u> GGC A K G V E A V M Y M G	20	pp1a	10	5551-5581	58
<u>TG</u> AAG TCA GAG <u>GAC</u> GCG <u>CAG</u> GGA <u>ATG</u> <u>GAT</u> K S E D A Q G M D	21	pp1a	9	6347-6375	79
<u>TT</u> <u>GAG</u> TGT AAT <u>GTG</u> AA E C N V	22	pp1a	4	6449-6464	88
<u>AGT</u> GGT <u>TTA</u> <u>GAT</u> TCT <u>TTA</u> <u>GAC</u> S G L D S L D	23	pp1a	7	7141-7159	57
<u>TA</u> GTT GCA GAG TGG TTT <u>TTG</u> <u>GCA</u> V A E W F L A	24	pp1a	7	7232-7254	61
<u>ATG</u> GTT <u>AGA</u> <u>ATG</u> TA M V R M	25	pp1a	4	7402-7416	79
<u>AT</u> GTA TGG AAA <u>AGT</u> TAT <u>GTG</u> CAT GTT <u>GTA</u> <u>GAC</u> GGT TGT A V W K S Y V H V V D G C	26	pp1a	12	7439-7477	72
<u>T</u> AGT <u>GAT</u> GAA GTT GCG AGA <u>GAC</u> TTG T S D E V A R D L	27	pp1a	8	7659-7684	69
<u>T</u> GAT GTT GGT <u>GAT</u> <u>AGT</u> GCG GAA GTT <u>GCA</u> GTT D V G D S A E V A V	28	pp1a	10	7998-8028	65
T TGT ATT <u>GAC</u> TGT <u>AGT</u> GCG C C I D C S A	29	pp1a	6	8334-8353	60
A TGG TTT <u>AGC</u> CAG CGT GGT <u>GGT</u> <u>AGT</u> T W F S Q R G G S	30	pp1a	8	8772-8797	58
<u>AT</u> GGT <u>GAC</u> TTT <u>TTG</u> CAT TT G D F L H	31	pp1a	5	8897-8915	53
<u>T</u> AGT GCA GTT <u>GGT</u> A S A V G	32	pp1a	4	8931-8944	86

<u>AT GAA AGT TTA CGC CCT GAC</u> E S L R P D	33	pp1a	6	9098-9117	70
<u>T AGT GGT AGA TGG GTA</u> S G R W V	34	pp1a	5	9264-9279	88
<u>TA GTA GCT GGT GGT ATT GTA GC</u> V A G G I V	35	pp1a	6	9404-9425	68
<u>ATG AGG TTT AGA AGA GCT TTT GGT GAA</u> M R F R R A F G E	36	pp1a	9	9457-9483	48
<u>AAG TTG CGT AGT GAT GTG</u> K L R S D V	37	pp1a	6	9838-9855	67
<u>AAG TAT TTT AGT GGA GCA ATG GAT</u> K Y F S G A M D	38	pp1a	8	9907-9930	75
<u>CA GCT GTT TTG CAG AGT GGT TTT AGA A</u> A V L Q S G F R	39	pp1a	8	10040-10067	52
<u>T GGT AAA GTT GAG GGT TGT ATG GTA</u> G K V E G C M V	40	pp1a	8	10083-10107	68
<u>T GGC ACA GAC TTA GAA GGT A</u> G T D L E G	41	pp1a	6	10572-10591	70
<u>AT GTT TTA GCT TGG TTG TAC GC</u> V L A W L Y	42	pp1a	6	10661-10682	45
<u>AT GGT ATG AAT GGA CGT A</u> G M N G R	43	pp1a	14	10874-10891	94
<u>T AGT TGG GTG ATG CGT ATT ATG ACA TGG TTG GAT ATG GTT GAT</u> S W V M R I M T W L D M V D	44	pp1a	6	11235-11277	70
<u>ATG TCA GAT GTA AAG TGC A</u> M S D V K C	45	pp1a	6	11848-11866	89
<u>AAG CTT TGT GAA GAA ATG CTG GAC</u> K L C E E M L D	46	pp1a	8	12049-12072	50
<u>ATG CAA CGT AAG TTG GAA AAG ATG GCT GAT</u> M Q R K L E K M A D	47	pp1a	10	12253-12282	70
<u>CAG GTT GTA GAT GCA GAT AGT A</u> Q V V D A D S	48	pp1a	7	12562-12583	86
<u>CAG GAT TTG AAA TGG GCT AGA T</u> Q D L K W A R	49	pp1a	7	12828-12849	77
<u>GC GGT ATG TGG AAA GGT TAT GGC TGT AGT TGT GAT</u> G M W K G Y G C S C D	50	pp1a	11	13382-13416	49
<u>C GGG TTT GCG GTG TAA GTG CAG CCC G</u> R V C G V S A A	51	pp1b	8	13466-13492	52
<u>TTT AAG TTT AGA ATA GAC GGT GAC ATG GTA</u> F K F R I D G D M V	52	pp1b	10	13743-13772	73
<u>AAG GAC TGG TAT GAT TTT GTA GAA</u> K D W Y D F V E	53	pp1b	8	13917-13940	58
<u>TA GGT GAA CGT GTA CGC CAA GCT TTG TT</u> G E R V R Q A L	54	pp1b	8	13972-13999	61
<u>TGT GAT GCC ATG CGA AAT G</u> C D A M R N	55	pp1b	6	14016-14034	74
<u>ACG CCA GGT AGT GGA GTT</u> T P G S G V	56	pp1b	6	14115-14132	83
<u>C AGA GAG CTA GGT GTT GTA</u> R E L G V V	57	pp1b	6	14483-14501	63
<u>TTT GTA GTT GAA GTT GTT GAT AAG TA</u> F V V E V V D K	58	pp1b	8	14850-14875	73
<u>TTT GAT TGT TAC GAT GGT GGC TGT A</u> F D C Y D G G C	59	pp1b	8	14877-14901	52
<u>A TGT GAT AGA GCC ATG CC</u> C D R A M	60	pp1b	5	15302-15319	83
<u>AA GTA TTG AGT GAA ATG GT</u> V L S E M	61	pp1b	5	15421-15439	74
<u>TT GCC GAT AAG TAT GTC CGC A</u> A D K Y V R	62	pp1b	6	15583-15603	62
<u>A TGT TGG ACT GAG ACT GAC</u> C W T E T D	63	pp1b	6	15833-15851	84
<u>A TGT GTT GGT AGC GAT AAT GTT</u> C V G S D N V	64	pp1b	7	16523-16544	64
<u>AT GCA TTG CCT GAG ACG ACA GCA GAT</u> A L P E T T A D	65	pp1b	8	17317-17342	81
<u>AA GAT TGT AGT AAG GTA</u> D C S K V	66	pp1b	5	18064-18080	71
<u>A AGA CAT GTA CGT GCA TGG ATT GGC</u> R H V R A W I G	67	pp1b	8	18278-18312	72
<u>AT GTA GCT AGT TGT GAT GCA</u> V A S C D A	68	pp1b	6	18841-18860	90
<u>AA GCT GAT GTA GAA TGG AAG TT</u> A D V E W K	69	pp1b	6	19066-19087	77

<u>AT GAT GCA CAG CCT TGT AGT G</u> D A Q P C S	70	pp1b	6	19090-19110	81
TTT TGG AAT <u>TGC AAT GTC GAT AGA T</u> F W N C N V D R	71	pp1b	8	19188-19212	56
TTG CCT GGT TGT <u>GAT GGT GGC AGT TTG TAT GTA</u> L P G C D G G S L Y V	72	pp1b	11	19270-19302	52
<u>AT GAG TAC AGA TTG TA</u> E Y R L	73	pp1b	4	19504-19519	94
AA <u>GTT GAT GGT GTT GAT GTA G</u> V D G V D V	74	pp1b	6	19720-19740	80
TTT TTT <u>GAT GGT AGA GTT GAT GGT</u> F F D G R V D G	75	pp1b	8	19983-20006	71
<u>T AGA AAT GCC CGT AAT GGT GTT</u> R N A R N G V	76	pp1b	7	20021-20042	55
TT TGG <u>TGT AAA GAT GGC CAT GTA GAA</u> W C K D G H V E	77	pp1b	8	20611-20636	50
A <u>AGT TGG ATG GAA AGT GAG TTC AGA GTT T</u> S W M E S E F R V	78	Gene S	9	22011-22039	69
<u>T AGA GGT GAT GAA GTC AGA C</u> R G D E V R	79	Gene S	6	22767-22786	85
<u>TG TAT AGA TTG TTT AGG AAG T</u> Y R L F R K	80	Gene S	6	22916-22936	70
CGG CGG <u>GCA CGT AGT GTA GCT AGT</u> R R A R S V A S	81	Gene S	8	23605-23628	75
TT GGA <u>AAG TAT GAG CAG TA</u> G K Y E Q	82	Gene S	5	25169-25187	68
<u>CAG CGA AAT GCA CCC CGC AT</u> Q R N A P R	83	Gene N	6	28297-28316	70
<u>CAG AAT GGA GAA CGC AGT GGG GCG CGA T</u> Q N G E R S G A R	84	Gene N	9	28354-28371	61
<u>AC GCA GAA GGG AGC AGA GGC GGC AGT</u> A E G S R G G S	85	Gene N	8	28786-28812	62
<u>CA GGC AGC AGT AGG GGA A</u> G S S R G	86	Gene N	5	28868-28885	72
<u>T AGA ATG GCT GGC AAT GGC GGT GAT G</u> R M A G N G G D	87	Gene N	8	28896-28921	65
C GGA <u>ATG TCG CGC ATT GGC ATG GAA GT</u> G M S R I G M E	88	Gene N	8	29217-29243	52

***ATG**, initio of the orf 1ab; %, percent of NTs-tCP conserved per RNA motif; double-underlined, represents repeated RNA motifs in the SARS-CoV-2 genome; NC, non-coding fragment; pp, polyprotein; N°, order of appearance in the genome. Notation: upper case letters represent NTs-tCP conserved in all beta-SARS-CoVs; underlined-bold upper case letters represent NTs-tCP conserved in most CoV genera; codons are represented by separated NT triplets in the correct reading frame, and amino acids tCP-conserved in most CoV genera are represented by bold upper case letters below their corresponding codons, amino acids tCP-conserved all beta-SARS-CoVs are represented by upper case letters below their corresponding codons.

Table 5. List of short RNA motifs and their associated peptides

Distribution of NTs-tCP conserved in short selected RNA motifs along the SARS-CoV-2 genome.

Differences in the frequency of appearance of NTs-tCP conserved in short selected RNA motifs were observed in Table 5. In order to gain a better sense of their distribution along the genome, Figure 4A shows a simple schematic of the genome organization of the SARS-CoV-2²⁸, together with the distribution of NTs-tCP conserved at the same scale (Figure 4B) in selected RNA motifs. When compared Figure 4A and 4B, it was observed the bulk of NTs-tCP conserved, located in the replicase-encoding orf 1ab, being the region with most NTs-tCP conserved and consequently the most populated with selected RNA motifs. The orf 1ab encode two overlapping polyproteins (abbreviated pp1a and pp1b) that regulate replication and transcription processes¹⁷ being then an optimum target for a possible therapy. The figure shows a comparative between NTs-tCP conserved when compared the SARS-CoV-2 and other SARS-CoVs (black), and the SARS-CoV-2 and other CoV genera (grey); differences observed in the frequency of appearance between both

cases are a consequence of differences in the number of tCPs conserved between the first and second cases (Table 4). The larger size motifs appear in the pp1a region following by the pp1b region. Four genome stretches of at least 500 bps do not contain any selected tCP motif in the pp1ab-encoding orf. One of them is located at 5' region and other in the middle of pp1a-encoding orf; there are two other in the middle of the pp1b-encoding orf. This means that despite the high similarity shown by this genome region, lacks of motifs tCP conserved in SARS-CoVs and also in other CoV genera. The spike-pp encoding-orf also contains broad regions non tCP conserved specially in the pp1b/spike interface. Many other genome regions lacking conserved tCP motifs as the protein-encoding orfs of the surface, the envelope and also the matrix (Figure 4B).

Figure 4. A) Genomic organization of SARS-CoV-2²⁸. B) Distribution of NTs-tCP conserved in human SARS-CoV-2 relative to other SARS-CoVs (black) and relative to most CoV genera (grey). The nomenclature as in Table 5.

To sum up, the results presented revealed that encoding proteins of SARS-CoV-2 as pp1a, pp1b, the spike polyprotein (Spp) and the nucleocapsid protein, contain tCP conserved fragments separated by genome fragments lacking tCP conservation. A high number of tCP conserved motifs were observed in the pp1ab-encoding orf. In contrast, fragments and genome regions lacking tCP conserved motifs were observed specially near the 5' regions of the genome.

Discussion

We will discuss whether or not some selected RNA motifs from Table 5 would have biological relevance. As it is not possible the analyses of all motifs from the table, we centre the attention on motifs 1, 9, 24, 39, 68, 79, 81 and 82, appearing in the literature inserted or attached to known relevant fragments of CoV genomes. It must be taken into account that conserved amino acids encoded by motifs of different CoV genera are there, attending possibly, to reasons of fitness or survival and could, therefore, be endowed with biological relevance.

The first point to be underlined, has to do with the fact that amino acids encoded by tCP-conserved codons would be tCP-conserved too; as a result (see Table 2), asparagine (D)-encoding

codons GAT and GAC, serine (S)-encoding codons AGT and AGC, valine (V)-encoding codon GTA, threonine (T)-encoding codon ACG, arginine (R)-encoding codon CGA, glutamine (Q)-encoding codon CAG, and alanine (A)-encoding codon GCA would be also conserved, as well as the initiation (ATG) and termination codons (TGA and TAG). Motif 1 would be an example of a highly conserved region surrounding the initio of pp1ab.

There are two identical RNA motifs, (TGTAGCTAGT), encoding the motif **V-A-S** (valine-alanine-serine). The motif appears in two regions of the SARS-CoV-2 genome, inserted in tCP conserved motifs 68 and 81(double-underlined), namely, (**AT GTA GCT AGT TGT GAT GCA**) and (CGG CGG **GCA CGT AGT GTA GCT AGT**), respectively; one is in the pp1ab-encoding orf and the other in the Spp-encoding orf. The **V-A-S** motif present in SARS-CoV-2 Spp, is absent, however, in other beta-SARS-CoV Spp's, namely, human SARS-CoV-1 and civet and bat SARS-CoVs; this is a notable difference between SARS-CoV-1 and SARS-CoV-2 genomes, suggesting that the motif found in the Spp would be species specific. The motif appears profusely in beta-SARS-CoVs pp1ab and also in alpha-HCoV pp1ab. Its presence is, however, very limited in other regions of CoV genomes (Supporting Table 1).

The motif **V-A-S** is attached to the R-R-A-R fragment of motif 81 (R-R-A-R-S-V-A-S) through a highly conserved serine, a cleavage site for the host protease furin necessary for activation of Spp of SARS-CoV-1 and MERS-CoV as proposed by a two-step sequential protease cleavage model^{29,30}; this support the importance of the motif in SARS-CoVs Spp because pp1a and pp1b help to release functional polypeptides by the papain-like protease (PL^{pro}) and the 3C-like protease (3CL^{pro}) situated in the non-structural protein (NSp) region, that could serve as substrate for some type of inhibitors. The short motif **V-A-S** appears often associated to SARS-CoV-1 3CL^{pro} cleavage sites, into canonical recognition positions; more specifically, at the same positions of NSp4/5 and NSp8/9³¹. Moreover, sequences containing **V-A-S** could be considered as potential substrates for some protease inhibitors in SARS-CoVs. The sequence of SARS-CoV-1 3CL^{pro} L-V-A-S-T, containing the **V-A-S**-motif, serve as substrate for classic protease inhibitors as 5-(2'-aminoethyl) aminonaphthalene sulfonic acid (EDANS)^{32,33}, amino benzoyl (abz)^{34,35}, or the colorimetric label pNA³⁴. It is the first time, as far as we know, that this sort motif has been suggested as a key fragment in certain experimentally deduced motifs having biological significance. This is supported by data describing that amino acid sequence alignment of SARS-CoV-2/SARS-CoV-1 replicase polyproteins show a high overall identity, resulting in a noticeable conservation at the polyprotein cleavage sites³⁶. The motif appears up to 10 times in the SARS-CoV-2 genome, 9 times in the pp1ab-encoding orf and one more time in the Spp-encoding orf, but only two of them are tCP conserved in all CoV genera, one in the pp1ab-encoding orf and the other in the Spp-encoding orf at positions 18841-18860 and 23605-23628, respectively (Table 5). A detailed analysis referred to the **V-A-S**-motif shows why information supplied by the tCP code-like is complementary to that obtained from the genetic code. As can be observed, the fact that the **V-A-S**-motifs are identical in SARS-CoVs does not imply necessarily they must be highly tCP-conserved in all CoV genera. In fact, there are notable differences in their tCP conservation, indicating, then, the evolutionary relevance of each one. The **V-A-S**-motif can be considered an example of how viruses incorporate simple motifs into their protein sequences to mimic human proteins and enhance their functional capabilities in host cells during infections. In addition, we suspect the **V-A-S**-motif could have a biological role in CoV genomes due to the high number of copies of the motif present in the pp1ab relative to the rest of viral proteins where few if any exist. Despite the size of the motif its presence in the pp1ab is much higher than expected relative to any other genome region (Supporting Table 1).

Other motif with possible biological relevance in beta-SARS-CoVs Spp could be the motif-82 encoding G-K-Y-E-Q, (TT GGA **AAG TAT GAG CAG TA**). It is located at the end of the Spp

and it is attached to the Spp fragment Y-I-K-W-P-W-Y-I-W-L, a highly conserved tryptophan W-rich membrane proximal external region (MPER) present on members of *Coronaviridae* family and more specifically in beta-SARS-CoV-2³⁷⁻⁴², forming the polypeptide G-K-Y-E-Q-Y-I-K-W-P-W-Y-I-W-L. Mutational studies have shown that W residues in the MPER are essential for effective viral infection and that MPER could serve as inhibitor for the entry process⁴³; the functional reason for the presence of the tCP conserved motif G-K-Y-E-Q attached to MPER would be related to the highly hydrophobic nature of the MPER peptide requiring the inclusion of the upstream charged residues K-Y-E-Q to increase its aqueous solubility⁴⁴.

Table 5 contains other repeated and conserved sequences in the SARS-CoV-2 genome, namely, (TTGCAGAGTGGTTTT) and (GAGTATGG), both located in the pp1a-encoding orf: The first one (double underlined), is inserted on selected RNA-motifs 24 and 39, namely, (TA GTT GCA GAG TGG TTT TTG GCA) and (CA GCT GTT TTG CAG AGT GGT TTT AGA A), respectively (Table 5). The peptides encoded by both RNA motifs are, V-A-E-W-F-L-A and A-V-L-Q-S-G-F-R, respectively. The peptide V-A-E-W-F-L-A appear related with the identification of potential cytotoxic T lymphocyte epitopes in the SARS-CoV-2 Indian isolate which can act as an effective vaccine epitope candidate⁴⁵; the peptide A-V-L-Q-S-G-F-R, could serve as a starting point for determining effective drug candidates for the treatment of SARS-CoV; the octapeptide naturally becomes an ideal good inhibitor for the SARS proteinase^{46,47}. The second repeated motif (double underlined), is inserted on selected RNA-motifs 8 and 10, namely, (GC ATT GAT TTA GAT GAG TGG AGT ATG GC) and (AT GAG TAT GGTA), respectively (Table 5). The peptides encoded by both RNA motifs are, I-D-L-D-E-W-S-M and E-Y-G, respectively; both are close together in the genome of SARS-CoV-2 (between positions 2945 and 3115), at the initio of the pp1a; they show 79% and 100% NTs-tCP conserved in all CoV genera, respectively. We have not found verified information in the literature about both motifs. However, the high level of tCP conservation between CoV genera make them good candidates for study.

The peptide (D-E-D-E-E-E-G-D-C-E-E-E-E-F-E) encoded by RNA motif 9, CA GAT GAG GAT GAA GAA GAA GGT GAT TGT GAA GAA GAA GAG TTT GAG CC (Table 5) has also interest. Segments of the motif have identity with functional fragments of the high mobility group box 1 (HMGB1) protein, actively secreted by inflammatory cells in response to pathogen-associated molecular patterns^{48,49} and probably related with SARS-CoVs pathology³⁹. An anti-HMGB1 monoclonal antibody has been developed⁵⁰ recognizing specifically the C-terminal tail of the motif 9, D-E-D-E-E-E. As can be observed, the motif contains a long acidic tail comprising, mostly glutamic (E) and aspartic (D) acids, similar to that described in HMGB1⁵¹. A current hypothesis is that immunization with anti-HMGB1 antibodies would confer protection against SARS-CoV-2 injuries⁵² by inhibiting the protease. Proteolytic processing of the CoV replicase is essential for ongoing viral RNA synthesis⁵³. The enzymatic activity of both PL^{pro} and 3CL^{pro} are essential for the viral life cycle. That is why, the beta-SARS-CoVs proteases are attractive targets for the development of antiviral drugs to reduce viral replication and pathogenicity⁵⁴.

The Spp is utilized by CoVs to bind to their cellular receptors. The crystal structure of the beta-SARS-CoV-2 S receptor-binding domain (RBD) interacting with the ACE2 receptor has been determined⁵⁵. The binding activates the fusion between the cell and SARS-CoV membranes for the virus entry into the cell^{55,56}. Into the SARS-CoV-2 RBD there is a receptor binding motif (RBM) containing most of the contacting residues for ACE2 binding⁵⁵. Inserted in the RBM, is the RNA motif 80, TG TAT AGA TTG TTT AGG AAG T-encoding the peptide Y-R-L-F-R-K (Table 5), highly conserved in beta-CoVs although less conserved in other CoV genera. We think the motif conserve the residues Y and R because they could be determinant for the effectiveness of the Spp/ACE2 interaction. We based the suggestion on the high percent identity of tCP-conserved NTs from other CoV genera (67%). Only five motifs, following tCP selection criteria,

have been selected in the Spp-encoding orf, and only one is located in the RBM. In the Spp RBM, the percent tCP-conserved residues between SARS-CoVs and other CoV genera is discrete, indicating that the protein motif would be specific of beta-SARS-CoVs; therefore, the relevance of the motif 80 is dictated by its location in the RBM and its implication in the Spp/ACE2 interaction. The Spp also contains the peptides R-R-A-R-S-V-A-S- and R-G-D-E-V-R encoded in RNA-motifs 81 and 79, namely, (CGG CGG GCA CGT AGT GTA GCT AGT) and (T AGA GGT GAT GAA GTC AGA C). Motif 79 is located in the RBD out of the RBM, and motif 81 is located out of the RBD, having 85% and 75% percent identity of tCP-conserved NTs, and also 67% and 75% tCP-conserved residues in other CoV genera different from beta-SARS-CoVs. Therefore, the V-A-S-fragment inserted in motif 81 appears again, now located in the SARS-CoV-2 Spp being relevant for two main reasons: first, it is known that effective conformational changes of S protein leading to membrane fusion, require both, the receptor binding and a proper protease activation. A R-R-A-R-furin site was found⁵⁵ in the S protein motif R-R-A-R-S-V-A-S between S1 and S2 subunits in SARS-CoV-2 S protein inserted in RNA motif 81 containing also the highly conserved V-A-S-fragment; second, this RNA-fragment found in the beta-SARS-CoV-2 S protein is absent, however, in human, civet and bat SARS-CoV S proteins, highlighting, thus, a fundamental difference between those CoVs in a CoV region specially sensible for the survival. It is known that the SARS-CoV Spp can be activated by host cell proteases, with proteolytic cleavage at the S1/S2 boundary and adjacent to a fusion peptide in the S2 domain⁵⁷. Thus, although elastase-mediated activation of SARS-CoV Spp has been considered an important factor for the severe pneumonia seen in SARS-CoV-infected patients, studies with neutrophil elastase (NE) failed to give any consistent activation of SARS-CoV S-mediated fusion⁵⁷. The V-A-S-motif appears also in the Spp and it is known that neutrophil elastase, prefers substrates containing the V-A-S-motifs at the P1 position. This fact endowing the V-A-S-motif even more evolutionary-relevance. Other factors such as the unique R-R-A-R-furin cleavage site at the S1/S2 boundary of the SARS-CoV-2 Spp could play a role in facilitating the rapid human-to-human transmission⁵⁵.

The MPER has an important role in the function of the S protein. The MPER, rich in aromatic residues with three or four W and two or three Y residues are present in all coronavirus S proteins^{43,58}. Such sequence conservation is also found in other viruses with class I fusion protein, such as human immunodeficiency virus type 1 (HIV1), feline immunodeficiency virus, influenza virus, and Ebola virus⁵⁹⁻⁶². The selected RNA-motif 82 (Table 5) attached to MPER acquire biological relevance principally for the following reasons: i) When working with synthetic peptides derived from CoV S proteins, the highly hydrophobic nature of the MPER peptide required the inclusion of upstream charged residues K-Y-E-Q in the peptide to increase its aqueous solubility and facilitating purification⁴⁴; ii) In SARS-CoV-2 Spp the upstream charged residues K-Y-E-Q-motif 82 are naturally attached to MPER (this paper); iii) K-Y-E-Q-Y-I-K shares sequential homology with the cholesterol recognition amino acid consensus motif⁶³, that was believed to result in a peptide or protein that preferentially associates with cholesterol, a principal component of lipid rafts⁴⁴.

The matrix protein M is an integral membrane protein involved in budding which interacts with the nucleocapsid and S proteins^{58,59}. As a component of the viral envelope plays a role in morphogenesis and assembly by interaction with other viral proteins. M protein is connected with infectivity through binding to S protein and the surface receptor, to promoting membrane fusion⁶⁰. In this context, the absence of the tCP-conserved V-A-S-motif in the beta-SARS-CoV-2 M protein and its presence in the beta-SARS-CoV M protein is, between others, one of the most notable differences between both human beta-SARS-CoVs. Supporting Table 1 illustrates that, since the point of view of the M protein, the V-A-S-motif, as it occurs with human SARS-CoV, is also present in civet and bat beta SARS-CoVs and also in human and porcine alpha-CoVs and

will be absent in the rest of CoVs. The presence of the **V-A-S**-motif in CoV Spp imply its absence in the matrix protein and vice versa, with one exception, the porcine alpha PEDV that having the motif in both. Moreover, the motif is present in SARS-CoV-2, murine MHV, porcine PEDV and avian CoV M proteins but not in SARS-CoV, civet and bat M proteins. The above data suggest more evolutionary proximity of civets and bats with human SARS-CoV with human SARS-CoV-2 that could be more evolutionary close to murine MHV. The presence of the **V-A-S**-motif in beta-SARS-CoV M protein could play a role in M protein because neutrophil elastase, having activity against a broad range of extracellular matrix proteins, prefers substrates with Val>Ala>Ser, Cys at the P1 position⁶¹.

The E (envelope) protein is the smallest of the structural proteins and it is abundantly expressed in the infected cell although, only a small portion is assimilated into the virion envelope⁶². Most M proteins are restricted at the site of subcellular trafficking where it participates in CoV assembly and budding⁶³. In SARS-CoV-2 the tCP-method does not selected any RNA motif for this small protein as occurred for the M protein. The only specie having a **V-A-S**-motif in the E protein was the porcine alpha PEDV (Supporting Table 1).

The SARS-CoV-2 N (nucleocapsid) protein is a RNA-binding protein playing vital roles in i) forming helical ribonucleoproteins, ii) regulating viral RNA synthesis in replication/transcription, and iii) modulating infected cell metabolism⁶⁴⁻⁶⁶, being a highly immunogenic and expressed protein during infection, inducing immune responses against human SARS-CoV and SARS-CoV-2^{67,68}. The most CoVs studied, with the exception of common moorhen delta-CoV (Supporting Table 1), lack the **V-A-S**-motif in the N protein, however, the tCP-method selected six RNA motifs conserved in beta-SARS-CoVs with percent of RNA conservation in other CoV genera ranging in between 52% and 72% (Table 4).

To sum up, the results presented revealed a new set of short RNA motifs in the SARS-CoV-2 by reading the genome fully-overlapping and categorizing RNA triplets by gross composition (Table 2). Some motifs show functional characteristics experimentally deduced and the remaining motifs must be taken into account in studies of functionality although today be unknown. For example, to our knowledge, the **V-A-S**-peptide, codified in motifs 68 and 81, has not been described before and however it is present in many protein fragments functionally relevant in CoVs. The same occur with other peptides as those codified in motifs 9, 68, 79, 80, 81, 82 (Table 5). In some cases, as the selected motif 80, it is inserted in a region functionally relevant as the RBM of the beta-SARS-CoV-2 S RBD to bind to the ACE2 receptor; in other cases, as the motif 82, it is attached to fragments functionally relevant, as the MPER, to increase its aqueous solubility and to facilitate its purification. It is unlikely to emerge those new motifs at random, inserted or flanking functional CoV genome fragments in general and in SARS-CoV-2 in particular. We suspect that selected RNA motifs could have significant roles in the fitness and survival of CoVs due their high degree of NTs-tCP conserved inter genera. They could be suitable for studies of inhibition and vaccines, more valuable because these motifs have not been observed heretofore.

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Author contributions

M.A.F. and C.A. designed this study and wrote the main manuscript text. M.A.F. analyzed the data and participated in data collection. Authors contributed to the discussion, edited the manuscript, and approved this manuscript.

Competing interests

The authors declare no competing interest

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