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Research paper

Therapeutic perceptions in antisense RNA-mediated gene regulation for COVID-19

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ABSTRACT

COVID-19 was first reported in Wuhan, China, in December 2019. It is widely accepted that the world will not return to its prepandemic normality until safe and effective vaccines are available and a global vaccination program has been successfully implemented. Antisense RNAs are single-stranded RNAs that occur naturally or are synthetic and enable hybridizing and protein-blocking translation. Therefore, the main objective of this study was to identify target markers in the RNA of the severe acute respiratory syndrome coronavirus, or SARS-CoV-2, with a length between 21 and 28 bases that could enable the development of vaccines and therapies based on antisense RNA. We used a search algorithm in C language to compare 3159 complete nucleotide sequences from SARS-CoV-2 downloaded from the repository of the National Center for Biotechnology Information. The objective was to verify whether any common sequences were present in all 3159 strains of SARS-CoV-2. In the first of three datasets (SARS-CoV-2), the algorithm found two sequences each of 21 nucleotides (Sequence 1: CTACTGAAGCCTTTGAAAAAA; Sequence 2: TGTGGTTATACCTACTAAAAA). In the second dataset (SARS-CoV) and third dataset (MERS-CoV), no sequences of size N between 21 and 28 were found. Sequence 1 and Sequence 2 were input into BLAST® ≫ blastn and recognized by the platform. The gene identified by the sequences found by the algorithm was the ORF1ab region of SARS-CoV-2. Considerable progress in antisense RNA research has been made in recent years, and great achievements in the application of antisense RNA have been observed. However, many mechanisms of antisense RNA are not yet understood. Thus, more time and money must be invested into the development of therapies for gene regulation mediated by antisense RNA to treat COVID-19 as no effective therapy for this disease has yet been found.

1. Introduction

COVID-19 was first reported in Wuhan, China, in December 2019 (Zhu et al., 2020). At the time of writing this paper, the pathological agent of COVID-19, namely the severe acute respiratory syndrome coronavirus, or SARS-CoV-2, has infected more than 33.2 million people and killed more than 1 million people worldwide. According to data from the World Health Organization, these numbers are continuing to increase. Patients with COVID-19 excrete viral nucleic acid at relatively high levels upon the onset of symptoms, and this excretion suggests transmissibility within a presymptomatic period that remains undefined (Koirala et al., 2020), making it difficult to track and control cases.

Abbreviations: Dec, Decimal; E, Envelope; EOF, End of File; M, Membrane; MERS-CoV, Middle East Respiratory Syndrome; miRNAs, microRNAs; mRNA, messenger RNA; N, Nucleocapsid; NCBI, National Center for Biotechnology; NS, Number Of Sequence; OI, Oral inhalation; ORF1ab, ORF1ab polyprotein; RNAi, RNA interference; S, Namely peak; SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus; siRNA, Small interfering RNA; TGS, Transcriptional gene silencing.

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Although social detachment and other strategies have reduced transmission and prevented many people from being infected, these strategies also leave people without immunity to SARS-CoV-2 and therefore susceptible to future waves of infection; healthcare professionals, the elderly, and people with underlying health problems are at particularly high risk (Chou et al., 2020). It is widely accepted that the world will not return to its prepandemic normality until safe and effective vaccines are available and a global vaccination program has been successfully implemented (DloC-cv, 2020).

In the 21st century, the *Coronaviridae* virus family gained three new species not previously identified. The first two new species were coronavirus with severe acute respiratory syndrome (SARS-CoV) in 2003 and coronavirus with Middle East respiratory syndrome (MERS-CoV) in 2012. Then, in 2019, a group of pneumonia cases was reported in relation to a wholesale seafood market in Wuhan. These cases were found to have been caused by a previously unidentified coronavirus (Jeyanathan et al., 2020). The new 2019 coronavirus was identified as 2019-nCoV in late December 2019 and later officially called SARS-CoV-2 (Committee ICoToVE, 2020). Coronaviruses are responsible for infections of the respiratory and gastrointestinal tracts (Su et al., 2016). Like other RNA viruses, the *Coronaviridae* family is characterized by high genetic variability and a high rate of recombination, both of which enable the virus to easily distribute itself among humans and animals. Of the existing coronaviruses in human and animal populations, many do not cause fatal diseases; however, the genetic recombination of viruses in random intermediate hosts can produce contagious strains that are highly pathogenic to humans. SARS-CoV-2 is genetically and structurally related to SARS-CoV but has unique characteristics that have contributed to its rapid spread around the world and a far more devastating global impact. (Tu et al., 2020). Understanding the structures of and proteins present in these viruses could enable the design of effective antiviral drugs (From, 2014; Calligari et al., 2020) and innovative therapies, such as small interfering RNA (siRNA) therapy (Liu et al., 2020).

As of April 6, 2020, 1,285,257 cases of COVID-19 in at fewest 170 countries and territories had been reported, with a corresponding mortality rate of approximately 5.4%. In China, an earlier investigation had reported 72,314 confirmed, suspected, and asymptomatic patients. Many of these confirmed cases (86.6%) were in the age group of 30 to 79 years. Of the 1,023 deaths, most were patients aged $\geq$ 60 years old, and the $\geq$ 80 age group was characterized by a mortality rate of 20.3%. A smaller number of cases among children aged 0–9 years were reported. In addition, the ratio of men to women affected by the disease varied depending on the population scale (1.06:1 in China, 1.04:1 in Hubei province, and 0.99:1 in Wuhan). Although COVID-19 is extraordinarily contagious, no deaths have occurred in mild cases, which make up the majority of all COVID-19 cases. As expected, lethality reached 49% among patients classified as critical cases (Chen et al., 2019). The common symptoms of COVID-19 are similar to those of SARS and MERS, namely fever (83%–98%), cough (59%–82%), shortness of breath (19%–55%), and muscle pain (11%–44%). Sore throat, rhinorrhea, headache, and confusion a few days before the onset of fever also affect some patients (Huang et al., 2019). COVID-19 is characterized by a clinical course that exhibits a wide spectrum of patterns in terms of severity and progression. In some patients, breathing difficulty is absent; however, dyspnea develops on average 8 days after the onset of the disease (interval: 5 to 13 days) (Wang et al., 2019). Of all COVID-19 patients, 3%–29% are admitted to intensive care units. Critically ill patients may experience rapid disease progression, multiple organ dysfunction, or even death (Wang et al., 2019). Furthermore, patients suffering from shortness of breath and hypoxemia may develop acute respiratory distress syndrome, severe sepsis with shock, or even multiple organ dysfunction within one week (Guan et al., 2019; Paraskevis et al., 2020).

The *Coronaviridae* virus family contains a single-stranded RNA genome of approximately 27–32 kb (Hoffmann et al., 2020). The organization of the genes is highly conserved, with the first being related to replication and transcription and the others being structural (de Wit et al., 2016). The SARS-CoV proteins consist of two large polyproteins, namely ORF1a and ORF1ab (which cleave proteolytically to form 16 nonstructural proteins); four structural proteins, namely peak (S), envelope (E), membrane (M), and nucleocapsid (N); and eight accessory proteins, namely ORF3a, ORF3b (NP_828853.1; not present in SARS-CoV-2), ORF6, ORF7a, ORF7b, ORF8a, ORF8b, and ORF9b (NP_828859.1; not present in SARS-CoV-2) (Tu et al., 2020; Liu et al., 2014). The scientific world is seeking an effective treatment and cure for COVID-19 as there is insufficient evidence to suggest that any existing antiviral drugs can effectively treat the disease. Several clinical trials to develop antiviral therapies are ongoing. Such therapies can be divided into two types, depending on their target. The first type is therapies that act directly on the coronavirus to inhibit the crucial viral enzyme responsible for the replication of the genome or to block the entry of the virus into human cells. The second type is therapies that modulate the human immune system by increasing its innate response, or in other words, by inhibiting the inflammatory processes that cause lung damage. Most of the drugs used in existing studies of COVID-19 were originally designed for other pathogens. However, several trials have been initiated to test vaccines and antibodies that specifically target SARS-CoV-2 (Tu et al., 2020). Many researchers around the world are committed to developing a vaccine for COVID-19, and at fewest 166 vaccine candidates are currently in preclinical or clinical development. However, because knowledge about the disease remains poor and the nature of protective immune responses is poorly understood, which vaccine strategies will be most successful remains unclear. Therefore, multiple vaccine platforms, strategies, and other forms of control must be developed in parallel (Jeyanathan et al., 2020). RNA interference (RNAi) is a mechanism by which siRNAs provide posttranscriptional gene silencing. This mechanism has yielded promising results in protecting against coronavirus viral invasion because it inhibits the expression of viral antigens and controls the replication and transcription of the viral genome (Wu and Chan, 2006). The role of RNAi in understanding immune cell modulation has been reported in (Mao et al., 2007) and (Dang et al., 2011).

SiRNA is a duplex RNA with nucleotides of 21–28 bases in length that is generated by the processing of long double-stranded RNA by the dicer enzyme. However, reportedly, nucleotides that are 21–22 bases in length effectively degrade messenger RNA (mRNA) (Elbashir et al., 2001). Therapy with siRNA has been efficient in the treatment of cancerous, viral, and genetic diseases (Liu et al., 2020). Great advances involving siRNA therapies for the treatment of other diseases have been proposed, such as the development of oral inhalation (OI) formulations based on propellants of siRNA-dendrimer complexes for the efficient silencing of pulmonary epithelium genes. Such developments are relevant because siRNA therapy is promising for the treatment of lung diseases (Conti et al., 2014). Antisense RNAs, unlike microRNAs (miRNAs) and siRNAs, are single-stranded RNAs that occur naturally or are synthetic and enable hybridizing and protein-blocking translation. Therefore, the main objective of this study was to identify target markers in the SARS-CoV-2 RNA with a length of 21–28 bases that enable the development of vaccines and drugs based on antisense RNA.

2. Methodology

2.1. *In silico analysis*

Three datasets composed of the sequences were downloaded from the National Center for Biotechnology Information (NCBI) website on May 30, 2020. The first set contained 3159 sequences of SARS-CoV-2 (taxid: 2697049; filter: Nucleotide completeness = “complete”). The second set consisted of 3471 sequences of SARS-CoV (taxid: 694009; filter: Nucleotide completeness = “complete”). The third set contained 529 sequences of the MERS-CoV (taxid: 1335626; filter: Nucleotide completeness = “complete”).

2.2. Method of analysis

A computational algorithm was developed in Language C (Kernighan and Dennis, 2006) to search for N nucleotide sequences that were repeated in all downloaded complete sequences. The algorithm is shown in Fig. 1. Our objective was to verify whether any common sequences were present in all strains of SARS-CoV-2 sequenced from the human host. The BaseConverter procedure, also shown in Fig. 1, was responsible for converting an N nucleotide sequence into an equivalent number in base 4 (formed by the 4 bases A, U, C, and G) so the value of the k position of the converted vector would be 0 for $k < N$ or a value representing the sequence included in the interval $[k - N + 1, k]$ for $k \geq N$. First, in an N nucleotide sequence composed of nitrogenous bases, each base was treated as a number between 0 and 3, namely A → 0, U → 1, C → 2, or G → 3. Then, the numeral resulting from the sequence was converted to a decimal number using Eq. (1), in which S is an array of size N formed by numerical values. As an example of the conversion process, assume a sequence of size 3 (UGC), or $S = \{1, 3, 2\}$; the decimal after the conversion is $4^2x1 + 4^1x3 + 4^\circ x2 = 2 + 12 + 16 = 30$; that is, the UGC sequence is equivalent to the number 30.

$$Dec(S) = \sum_{i=1}^{N} 4^{N-i} * S \quad (1)$$

After all the N nucleotide sequences in a complete sequence were converted, the converted vector was ordered by the Sort procedure. Sorting was performed using the QuickSort method (Hoare, 1962) because it is the most efficient sorting method available. After the vector was ordered, it was analyzed using the Search1 or SearchN method, depending on its index in the dataset (NS). In Search1, the first and second complete sequences of the dataset were compared by checking whether a given value in the first complete sequence was also present in the second complete sequence; this procedure was performed for each value in the first complete sequence using a binary search (Knuth, 1971). If a certain value was present, the number of times it was repeated was checked, and that value and the corresponding number were stored. The SearchN procedure checked whether the values found in Search1 were also present in the other complete sequences in the dataset. This procedure was performed until all the complete sequences had been analyzed for each value found in Search1 through a binary search.

Finally, the values found in all complete sequences in the dataset were converted to base 4, and all discovered N nucleotide sequences were returned. To illustrate this process, Fig. 2 shows a fictitious dataset containing four complete sequences, all of size 8, and the process is guided by the search for sequences of three nucleotides (N = 3). In step 1, the first two complete sequences were converted using the BaseConverter procedure. Then, the Sort procedure ordered converted vector number 2. Finally, the Search1 procedure checked whether any value contained in converted vector number 1 was also present in vector 2; all values contained in both vectors as well as the number of times they appeared were stored. In step 2, the third complete sequence was converted by the BaseConverter procedure, and the converted vector was ordered using Sort. Then, the SearchN procedure checked which values stored in step 1 were also present in the third complete sequence of the dataset. Finally, in step 3, the same process was conducted for the fourth and final complete sequence. Upon completion of the search, the stored sequences of three nucleotides were converted to base 4 and returned as a response.

The use of a binary search for the ordered vectors made it possible to decrease the computational processing time in comparison with the exhaustive searches involved in large datasets such as that used in this study. Based on reports of the silencing efficiency of siRNAs with 21 to 28 bases (Elbashir et al., 2001), we used N with a value between 21 and 28.

2.3. Identification of sequences found in the in silico analysis

To identify the nucleotide sequences found by the computational algorithm, we used the NCBI-BLAST® ≫ blastn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE = BlastSearch&BLAST_SPEC = Betacoronavirus). This platform offers current information and is continuously updated according to scientific advances related to SARS-CoV-2. As mentioned, the sequences found by the algorithm were two sequences each of 21 nucleotides (Sequence 1: CTACTGAAGCCTTTGAAAAAA; Sequence 2: TGTGGTTATACCTACTAAAAA). These sequences were input into BLAST® ≫ blastn suite for identification. To identify the strings, we analyzed the values of their specificity using the following functions: Query cover, E-Value, Expect and Identities.

3. Results

In the first dataset (SARS-CoV-2) the algorithm found two sequences of 21 nucleotides (Sequence 1: CTACTGAAGCCTTTGAAAAAA; Sequence 2: TGTGGTTATACCTACTAAAAA). In the second (SARS-CoV) and third (MERS-CoV) datasets, no sequences of N = 21–28 were found. We then verified in how many complete sequences the two sequences of 21 nucleotides found in the first dataset appeared in the SARS-CoV and MERS-CoV datasets. In the SARS-CoV dataset, Sequence 1 appeared 3160 times, and Sequence 2 appeared 3159 times. In the MERS-CoV dataset, neither sequence appeared. Because the SARS-CoV dataset contained the SARS-CoV-2 dataset, we concluded that the two sequences of 21 nucleotides appeared in the SARS-CoV-2 virus code but not in the SARS-CoV or MERS-CoV virus codes. Sequences 1 and 2 were input into BLAST® and recognized by the platform. Subsequently, both sequences were identified based on their specificity using the following functions: Query cover, E-Value, Expect, and Identities. The gene identified by the sequences found in the developed algorithm was the ORF1ab region of SARS-CoV-2, as shown in Tables 1 and 2.

4. Discussion

The scientific world is currently in search of an effective vaccine or therapy against COVID-19; however, no such vaccine or therapy has yet

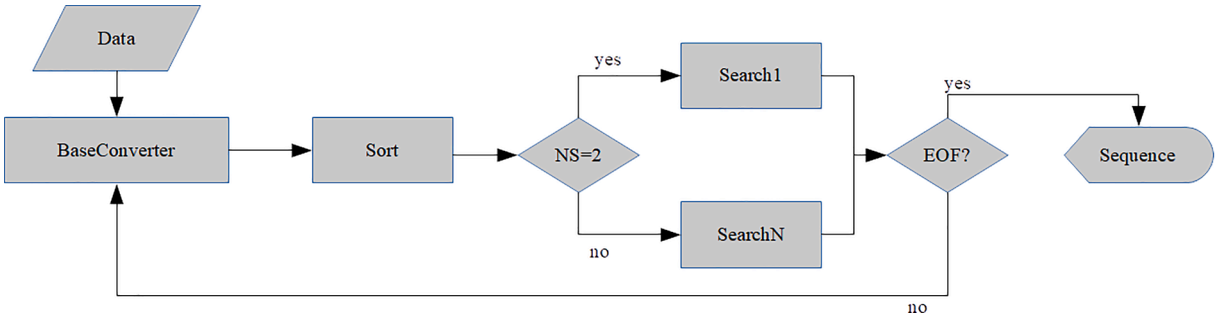

Fig. 1. Main flowchart. NS: number of the complete sequence being analyzed. EOF: End of File, indicating that all complete sequences have been analyzed.

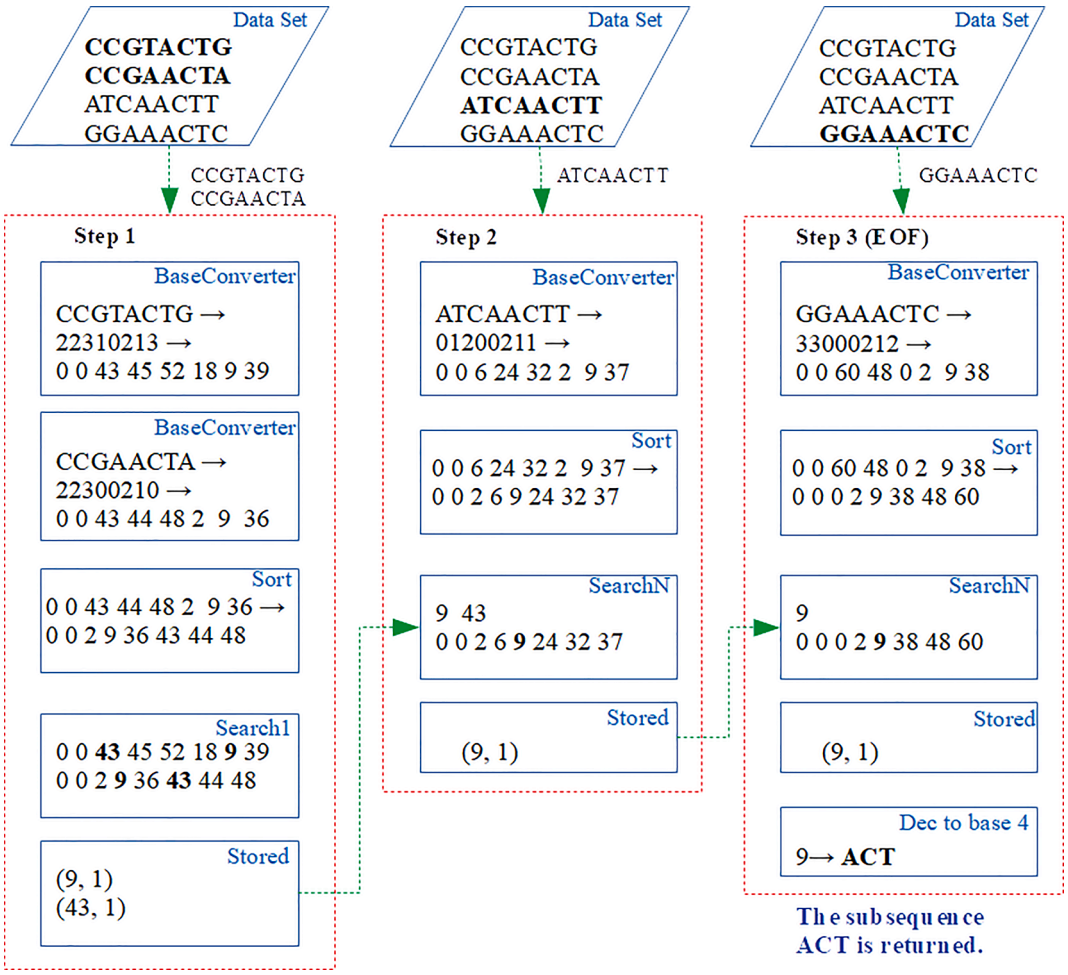

Fig. 2. Example of the general search procedure.

been found. At the time this paper was written, at fewest 166 vaccine candidates were under preclinical or clinical development. However, because knowledge about the disease remains scarce and the nature of protective immune responses is poorly understood, which vaccine strategies will be most effective remains unclear. Therefore, multiple strategies to control the spread of the disease must be developed in parallel (Jeyanathan et al., 2020). Over the preceding decade, siRNAs have been shown to functionally modulate epigenetic states in human cells to achieve functional transcriptional gene silencing (TGS). The discovery of siRNA following those of TGS and RNAi undermined the early studies of TGS and thus was neglected. TGS is mechanically distinct from the RNAi gene silencing pathway and can result in long-term stable epigenetic changes in gene expression that can be transmitted to daughter cells during cell division. By contrast, such changes do not occur with RNAi. Non-coding RNA plays a role in regulating transcription and silencing the epigenetic gene in human cells. Studies related to TGS have indicated that functional TGS may be the basis for the development of future therapeutic agents (Weinberg and Morris, 2016).

SiRNA, miRNA, long noncoding RNA, PIWI interaction RNA, and noncoding RNA have become popular research topics in relation to diseases such as cancer, various viruses, lung diseases, and genetic disorders (Liu et al., 2020). MiRNAs regulate gene expression through base complementarity between mRNAs and miRNAs rather than through mRNA degradation (Schmiedel et al., 2015). However, the combination of double-stranded RNAs and miRNAs triggers the degradation of mRNA. Thus, miRNAs are fundamental in mediating the degradation of mRNA in the region of 20 base pairs in length and perform several functions to regulate the expression of coding genes (Mohr and Mott, 2015). SiRNA is a class of double stranded noncoding RNA that is 20–25 base pairs in length. SiRNAs can regulate gene expression, and therapy with siRNAs has proven effective for the treatment of cancerous, viral, and genetic diseases (Liu et al., 2020). SiRNA therapies for treating other diseases have been proposed, such as the development of OI formulations based on propellants of siRNA-dendrimer complexes for the efficient silencing of pulmonary epithelium genes. Such developments are relevant because siRNA therapy is promising for the treatment of lung diseases (Conti et al., 2014). Antisense RNAs, unlike microRNAs and siRNAs, are single-stranded RNAs that occur naturally or are synthetic and enable hybridizing and protein-blocking translation. Furthermore, antisense RNAs are unique DNA transcripts. They are small, noncoding, diffusible molecules containing between 19 and 23 nucleotides that complement mRNA. A few decades ago, antisense RNA was considered a waste of RNA; however, today, it is recognized as an extraordinary material with high potential for the regulation of intracellular genes. Antisense RNA is a unique form of noncoding RNA used to regulate genetic activity at multiple levels in cells; such activity includes that related to DNA, RNA, chromosomal structures, transcription, translation, and protein stability (Rusk, 2014). With development, antisense RNAs could replace traditional technologies for gene silencing. Thus, antisense RNAs constitute a novel therapeutic approach (Zeng et al., 2019).

Using the described algorithm, we found two sequences each with 21 nucleotides (Sequence 1: CTACTGAAGCCTTTGAAAAAA; Sequence 2: TGTGGTTATACCTACTAAAAA). These strings were input into BLAST® ≫ blastn and recognized by the platform. Subsequently, they were

Table 1
BLAST results for nucleotide Sequence 1 (CTACTGAAGCCTTTGAAAAAA) obtained with NCBI BLAST® » blastn suite.

Gene:	ORF1ab
Protein title:	ORF1a polyprotein
Protein title:	ORF1ab polyprotein
Merged features:	
[Length]	QOE45358.1 and QOE45357.1
Protein length:	
	4,405 aa
CDS length:	21,291 nt
Protein length:	
[QOE45358.1]	7,096 aa
CDS position:	11,700
Protein position:	3,900
Protein sequence:	
	SKLWAQCVQLHNDIL[L]AKDTTEAFEKMVSL
[QOE45357.1]	
CDS position:	
	11,700
Protein position:	
	3,900
Protein sequence:	
	SKLWAQCVQLHNDIL[L]AKDTTEAFEKMVSL
Links and tools	
BLAST protein:	QOE45358.1
	QOE45357.1
BLAST nr:	MW049023.1 (261.0.21,550)
FASTA record:	MW049023.1 (261.0.21,550)
GenBank record:	
	MW049023.1 (261.0.21,550

Table 2
BLAST results for nucleotide Sequence 2 (TGTGGTTATACCTACTAAAAA) obtained with NCBI BLAST® » blastn suite.

Gene:	ORF1ab
Protein title:	ORF1a polyprotein
Protein title:	ORF1ab polyprotein
Merged features:	
[Length]	QOE45358.1 and QOE45357.1
Protein length:	4,405 aa
CDS length:	21,291 nt
Protein length:	
[QOE45358.1]	7,096 aa
CDS position:	3,884
Protein position:	
	1,295
Protein sequence:	
	KKDAPYIVGDVVQEG[V]LTAVVIPTKKAGGT
[QOE45357.1]	
CDS position:	
	3,884
Protein position:	
	1,295
Protein sequence:	
	KKDAPYIVGDVVQEG[V]LTAVVIPTKKAGGT
Links and tools	
BLAST protein:	QOE45358.1
	QOE45357.1
BLAST nr:	MW049023.1 (261.0.21,550)
FASTA record:	
	MW049023.1 (261.0.21,550)
GenBank record:	
	MW049023.1 (261.0.21,550

identified with high accuracy in the ORF1ab region of SARS-CoV-2 (Tables 1 and 2). Based on scientific advances toward antisense therapy, a proposal for an effective treatment for COVID-19 could be a therapy based on an antisense RNA to block the translation of a protein from SARS-CoV-2. Given the high amount of research on SARS-CoV-2 currently being conducted, the question of which target is more viable to block the function and pathogenicity of COVID-19 is worthy of consideration. Systematic knowledge of the proteins present in SARS-CoV-2 could enable the development of a smart approach for creating effective antisense therapies. The SARS-CoV proteins are composed of two large polyproteins, namely the ORF1a and ORF1ab regions (which cleave proteolytically to form 16 nonstructural proteins); four structural proteins, namely peak (S), envelope (E), membrane (M), and nucleocapsid (N); and eight accessory proteins, namely ORF3a, ORF3b (NP_828853.1; not present in SARS-CoV-2), ORF6, ORF7a, ORF7b, ORF8a, ORF8b, and ORF9b (NP_828859.1; not present in SARS-CoV-2) (Liu et al., 2014; Yoshimoto, 2020). The literature reports that viral S protein subunit vaccines produce high quantities of neutralizing antibodies and more comprehensive protection than live attenuated SARS-CoV or complete protein S and DNA-based protein vaccines (Buchholz et al., 2004). At the time this paper was written, half of all patents for COVID-19 vaccines under development were aimed at protein vaccines comprising the S protein subunit vaccine and vaccines that specifically target the S1 subunit receptor-binding domain of the viral S protein. The protein, or S gene, is the preferred target site in the development of a SARS-CoV-2 vaccine. In our in silico analysis, the sequences found were recognized as those of the ORF1ab protein. We believe that owing to the importance of the ORF1ab region, this region could be a suitable target for the introduction of an antisense therapy. Antisense RNAs can be used as effective regulators of genetic activity at multiple levels. Thus, they are fast becoming the focal point of many genetic studies

5. Conclusion

Antisense RNA is recognized as a powerful new tool for protection against COVID-19 (Viruses, 2014). Considerable progress in antisense RNA research has been made in recent years, and great achievements in the application of antisense RNA have been observed. However, many mechanisms of antisense RNA are not yet understood. Thus, more time and money must be invested into the development of therapies for gene regulation mediated by antisense RNA to treat COVID-19 as no effective therapy for this disease has yet been found.

CRediT authorship contribution statement

Sabrina Ferreira Jesus: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - original draft. **Laércio Ives Santos:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - original draft. **João Felício Rodrigues Neto:** Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing - original draft. **Thallyta Maria Vieira:** Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing - original draft. **João Batista Mendes:** Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Writing - original draft. **Marcos Flavio Silveira Vasconcelos D'angelo:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Writing - review & editing. **André Luiz Sena Guimaraes:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Validation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Dr. Guimarães, is research fellows of the CNPq.

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