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Original Article
Özgün Araştırma

Isolation of SARS CoV-2 and evaluation of human-animal cases

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Abstract: Complete genome analyses of SARS CoV-2 isolated from three Turkish patients are compared with other complete genome sequences in the world. In this study, especially the sequence data from animals were also involved in the evaluation. When the genetic data collected from animal and human COVID-19 cases were analyzed, it was evaluated that some recent nucleotide changes in human cases were similar to those of some animal COVID-19 cases. It is recommended that nucleotide or protein changes in human cases in SARS CoV-2 be followed and compared with large-scale studies in animals.

Keywords: SARS CoV-2, Turkey, Animal, COVID-19, Veterinary

SARS CoV-2 izolasyonu ve insan hayvan vakalarının değerlendirilmesi

Özet: Üç hastadan izole edilen SARS CoV-2'nin tam genom analizleri dünyadaki diğer tam genom dizileriyle karşılaştırılmıştır. Bu çalışmada özellikle hayvanlardan alınan dizilim verileri de değerlendirmeye dahil edilmiştir. Hayvan ve insan COVID-19 vakalarından toplanan genetik veriler analiz edildiğinde, insan vakalarında yakın zamanda meydana gelen bazı nükleotid değişikliklerinin bazı hayvan COVID-19 vakalarındakine benzer olduğu değerlendirildi. SARS CoV-2'de insan vakalarındaki nükleotid veya protein değişikliklerinin hayvanlarda yapılacak geniş çaplı çalışmalarla takip edilerek karşılaştırılması önerilmektedir.

Anahtar kelimeler: SARS CoV-2, Türkiye, COVID-19, Hayvan, Veteriner

Introduction

The COVID-19 outbreak has now reached over 510 million confirmed cases and 6,2 million confirmed deaths worldwide since 2019 (WHO 2022) .

Coronaviruses belong to the *Orthocoronavirinae* subfamily of the *Coronaviridae* family in the order *Nidovirales* (Gorbalenya et al. 2020). There are four genera in the subfamily: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*. Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) are important human pathogens in betacoronaviruses (Park 2020). According to genome sequencing, SARS-CoV-2 is a new member of betacoronaviruses (Zhu et al. 2020).

The symptoms of COVID-19 are like respiratory illnesses caused by respiratoric viruses such as other coronaviruses and influenza viruses. So, these diseases are mostly confused with each other. While the most common symptoms of COVID-19 are fever, dry cough, tiredness, diarrhea, muscle or body aches and pains, conjunctivitis, loss of taste or smell, and headache, more serious symptoms are difficulty in breathing or shortness of breath, chest pain or pressure, inability to wake up or stay awake, bluish lips or face (Rabaan et al. 2020; CDC 2020).

After the first SARS CoV-2 cases, researchers started to investigate SARS CoV-2 to find out its source. To date, there is not enough evidence about the source of SARS CoV-2. On the other hand, genetic sequence data of *Rhinolophus* (horseshoe bat) and pangolin coronaviruses are closely related to SARS CoV-2 in human cases. Scientists are carrying out studies about SARS CoV-2 to find the original route of transmission to humans (OIE 2021; Lam et al. 2020).

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According to the World Organisation for Animal Health (OIE 2020) ferrets, American mink (*Neovison vison*), racoon dogs (*Nyctereutes procyonoides*), domestic cats, large cats (tigers, lions and puma), Egyptian fruit bats (*Rousettus aegyptiacus*) and Golden Syrian hamsters (*Mesocricetus auratus*) could transmit SARS CoV-2 between their populations. Only American minks could transmit SARS CoV-2 to humans (OIE 2020). Ferrets, American minks, dogs, domestic and large cats could show clinical signs. A lot of countries have been affected by the morbidity and mortality of SARS CoV-2 disease in mink farms. SARS CoV-2 incubation period and symptoms seem similar in animals and humans but more studies required to clarify (OIE 2021; OIE 2020)

Transgenic mice, Rhesus macaques, juvenile cats, ferrets and rabbits show similar SARS CoV-2 lesions or histopathological findings like humans such as interstitial pneumonia, inflammatory cell infiltration around the bronchioles and blood vessels, massive lesions in the nasal and tracheal mucosa epithelia and lungs (OIE 2020; Cohen 2020; Schlottau et al. 2020).

SARS CoV-2 infected people should stay in quarantine during their illness to prevent the transmission to other people. Similarly, people should implement the same restriction conditions for mammalian animals, including pets (OIE 2020; OIE 2021).

SARS CoV-2 complete genome analyses isolated from three Turkish patients are compared to other complete genome sequences from around the world. In this study, the sequence data from animals, in particular, were evaluated. When genetic data from animal and human COVID-19 cases was analyzed, it was discovered that some recent nucleotide changes in human cases were similar to those in animal COVID-19 cases.

Material and Method

Real Time RT-PCR

For the isolation of SARS CoV-2, RNA extraction (QIAamp cador Pathogen Mini Kit) was performed from the swab samples collected from the respiratory tract of eight people treated with the diagnosis of SARS CoV-2 in 29 Mayıs State Hospital affiliated to the Ministry of Health of Turkey. SARS CoV-2 test was performed by Real Time RT-PCR method on Biorad CFX-96 device using primary sequences recommended by the World Health Organization (Corman et al. 2020) and QuantiNova Pathogen + IC Kit (Qiagen, Hilden, Germany). Only three samples were found positive in the tests. Positive samples were cultivated into Vero E6 cell culture using the adsorption method.

Virus Culture

Vero E6 cells seeded in 25 cm^3 cell culture flasks were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma, United States). Samples were filtrated from 0.22 µm syringe filters (Merck, Germany) every time before inoculating to cell culture and incubated for 1 h at 37 °C and 5% CO_2. The inoculum was then removed and replaced with fresh DMEM. After incubation, cells were observed every day for the presence of a cytopathic effect (CPE) under the microscope, and culture supernatants were harvested, aliquoted, and stored at -80°C.

Afterwards, inoculation and nucleic acid extraction procedures were applied to the cell culture again. Real Time RT-PCR test was applied to the extractions as stated above. In this way, 3-5 passages were made and the samples that were cultivated each time were confirmed to be positive. Samples with codes ETLKVET1, ETLKVET2 and ETLKVET3 were isolated. It was transferred to the next stage for full genome analysis.

MinION Sequencing

Sequencing steps and Bioinformatic data were prepared by a company named Nucleus Genetics (İzmir, Turkey). Library preparation for the MinION sequencing was performed using the Ligation Sequencing kit SQK-LSK109 and Natives Barcoding based on the manufacturer's instructions and modifications.

MinION Bioinformatics Workflow

nCoV-19 Genome Analyzer was used for SARS CoV-2 genome analyzing. Selected reads were mapped against SARS-CoV-2 reference (NC_045512) using Minimap2 (v2.9) (Li 2018). SAMtools (v1.9) were used to sort the aligned BAM files to obtain coverage data and a consensus sequence. Alignment statistics were also calculated with SAMtools.

Phylogenetic analyses

In order to perform whole genome analysis, 126 full genome data of SARS CoV-2 were acquired from GSIAD and GenBank. While selecting human samples, priority was given to having samples from

every continent and the countries that have most cases in the continents. All full genome SARS CoV-2 data we encounter in animals in two databases were selected. Our three samples (ETLKVET1, ETLKVET2 and ETLKVET3) were added to these samples.

Total of 129 full genome SARS CoV-2 sequences were aligned using the ClustalW (Larkin et al. 2007) algorithm.

Phylogenetic analysis was performed based on the maximum likelihood (ML) criteria by using MEGA X (Kumar et al. 2018), MrBayes (Ronquist and Huelsenbeck 2003), RAxML (Stamatakis 2014), iQ-TREE (Nguyen et al. 2015), PHYLIP (Felsenstein 2005) and PAUP 4.0 (Swofford 2003) programs and the accuracies of the trees were checked. Similar trees were acquired in all the programs used.

Subsequent models were developed that incorporate differences for all relative rates to the general time reversible gamma model (GTR+G model). By GTR + G method, 1000 bootstrap was made to find the most suitable tree in MEGAX, RAxML, PAUP 4.0 and IQ-TREE programs.

In the MrBayes program, using by the GTR + G method with Markov chain Monte Carlo (MCMC) analysis, the most suitable tree was found by performing bootstrap in 2 different channels until the cluster value was below 0.001. 14 million MCMC chain iterations were sampled every 100 generations, corresponding to 22 000 trees for SARS CoV-2 spike nucleotide analyses. 5 million MCMC chain iterations were sampled every 100 generations, corresponding to 50 000 trees for SARS CoV-2 full genome nucleotide analyses. In MCMC analyses, the mixture of samples was controlled with Tracer 1.3 (Rambaut et al. 2018).

The data from the programs were arranged using Figtree 1.4.3 (Rambaut 2009) and Dendroscope 3 (Huson and Scornavacca 2012) softwares.

Results

Virus growth was observed in three of the eight specimens inoculated in cell culture. It was checked daily in terms of CPE.

PHYLIP, MEGA X, RaXML, MrBayes, PAUP 4.0a168, IQTREE programs were used to verify the reliability of phylogenetic trees. Since the results acquired in all programs were close to each other, the trees were interpreted to be reliable.

Nucleotide and amino acid changes were examined by downloading the SARS CoV-2 full genome data acquired from animals and randomly selected people from each continent in GenBank and GSIAD.

Nucleotide and amino acid changes (Orf, Spike) varied by 1-0.5%.

Full genome analysis

When the tree created using the full genome was examined, it was seen that although most of SARS CoV-2 sequences acquired from animals formed a group, some of them were intertwined with those obtained from humans (Figure 1).

It was seen that our ETLKVET2 coded sample was in the same branch with Turkey ACUTG-2, Saudi Arabia 29903 and Saudi Arabia KAUST samples.

When ETLKVET1 and ETLKVET3 were evaluated in general, it was seen that they were closely related to SARS CoV-2 detected in many countries.

Comparing the data acquired from animals and humans, it was seen that USA feline MT425184 detected in cats in USA and MT259226 detected in human in China were in the same branch.

KUMCO1 detected in human in South Korea and those detected in Panthera leo in USA (MT704312, MT704311, MT704310, MT747978, NY 041520, MT425183) are in the same branch with the coded sequence as MT215193 detected in dog in Hong Kong. However, sequences detected from canine and feline species were also found in the same branch (Figure 1).

The mutated SARS CoV-2 detected in England in December 2020 are in the same branch with sequences detected in Felis catus (MT709104, MT709105) in France, sequences detected in many minks in Denmark and GLAB-cov217 identified from Turkey. These mutated SARS CoV-2 were detected in minks between May and November and it was detected before human cases.

Although SARS CoV-2 sequences found in mink in the Netherlands formed a group within themselves, Tygerberg 07 2020 and Nigeria ED32 CV164 sequences acquired from people from South Africa and Nigeria also joined this group.

The sequences detected in Panthera tigris in USA (tiger NY P3, MT704314, MT704315, MT704316, tiger NY 4, MT704317, MT704313, MT365033), and sequences detected in humans and named as Australia VIC1658, New Zealand 29782 and Georgia Tb 7856 were detected in the same branch (Figure 1).

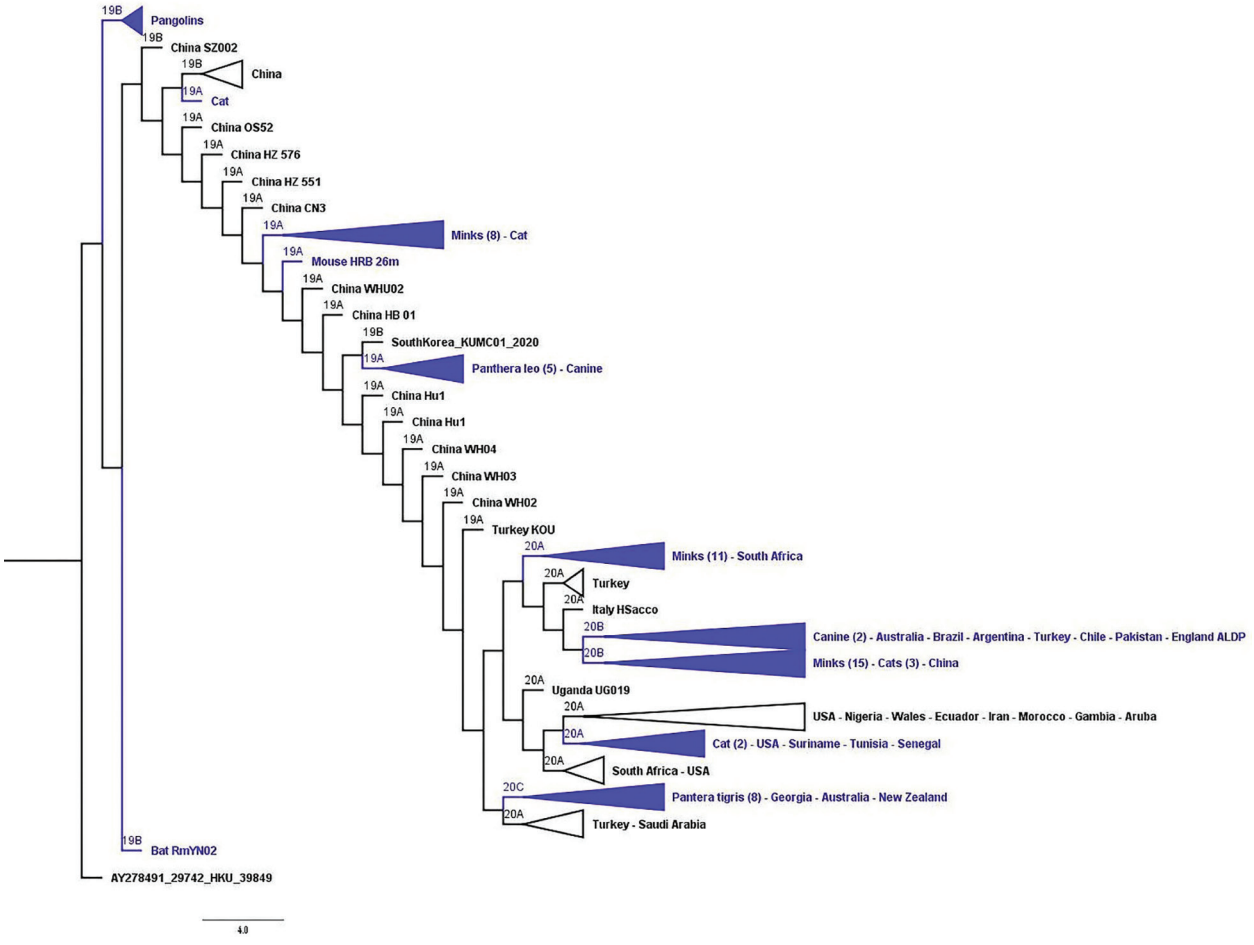

Figure 1. Phylogenetic analysis of full genome of SARS CoV-2. Blue coloured branches indicated SARS CoV-2 sequences from animals and colourless branches indicated SARS CoV-2 sequences from humans. Genetic clades of SARS CoV-2 were shown on the branches

Trees were also created for protein-coding regions. Other than spike, no significant difference was found. The tree created for the spike is presented in detail due to its roles in antibody response and virus entry into the cell (Figure 2). In trees made of both full genome and spike protein, SARS CoV-2 detected in human, canine, mink, leopard, tiger and mouse, except for SARS CoV-2 detected in bats and pangolins in Wuhan, were found in a single main branch. The findings acquired from this tree are as follows;

Turkey ETLKVET1 and ETLKVET2 (April 2020) sequences were closely related to Denmark mink MT919536 (June 2020) and Belgium Felis catus MT747438 (March 2020) sequences. ETLKVET3 was first found close to Ukraine 203100352 and then to SARS CoV-2 detections in many countries.

It was seen that human samples with codes Gambia NPHL 2374 (May 2020), USA WT UW 535 (June 2020), New Zealand 29782 (May 2020) and Georgia 7856 (May 2020) were closely related to the canine Hong Kong 29764 (March 2020), USA Panthera Tigris MT704313 and MT365033 (April 2020), Denmark mink MT919535 (June 2020) and MT99531, and France Felis catus MT709105 (May 2020). It was seen that these tree branches have close relationship with SARS CoV-2 sequences acquired from animals (mink DK AL3, Felis catus FRA and tiger NY 040420). Again, in another branch, Belgium cat MG0320 (2020 March), France Felis catus MT709104 (May 2020) seemed closely related to Saudi Arabia 29903 (May 2020), China Zhejiang SX0715 (July 2020) and Turkey KOU MG (May 2020).

USA Panthera Tigris MT704317 (April 2020) and France cat Env Di 483064 (May 2020) with USA Tx

HM0306 (March 2020), Uganda UG019 (April 2020), Wales PHWC 36C94 (April 2020) and Italian env LOM INMI HSacco (April 2020) seemed to be closely related.

It was seen that the genetic data detected in the canine (MT215193) at the beginning of the epidemic in Hong Kong and in China were also close to each other.

England ALDP C4812B coded sequence which was first detected in the UK in December 2020 was in the same branch with tiger USA NY (April 2020) and Panthera tigris MT704316 (April 2020). Examination results of the tree obtained in terms of spike protein were also evaluated in this way.

In the table obtained from the nucleotide changes (Table 1), it is seen that the sequences belonging to animals form 3 groups different from those of humans. These are formed by the samples of Georgia, Australia, New Zealand along with minks, Panthera leo and Panthera tigris.

Although many nucleotide changes are seen in different samples in the genetic sequence of SARS CoV-2, most of them do not change the amino acid synthesis.

Among the sequences evaluated, there are 7 amino acid changes seen only in mink. Likewise, only 7 amino acid changes were seen in Panthera leo group different from the others. There are human data available from three other countries with Pantera tigris. In this group, 2 amino acid changes were also observed. Unlike other groups, the changes seen in animals here were also detected in humans.

The data obtained from animals in trees are marked in red and it is seen that these are concentrated on certain branches.

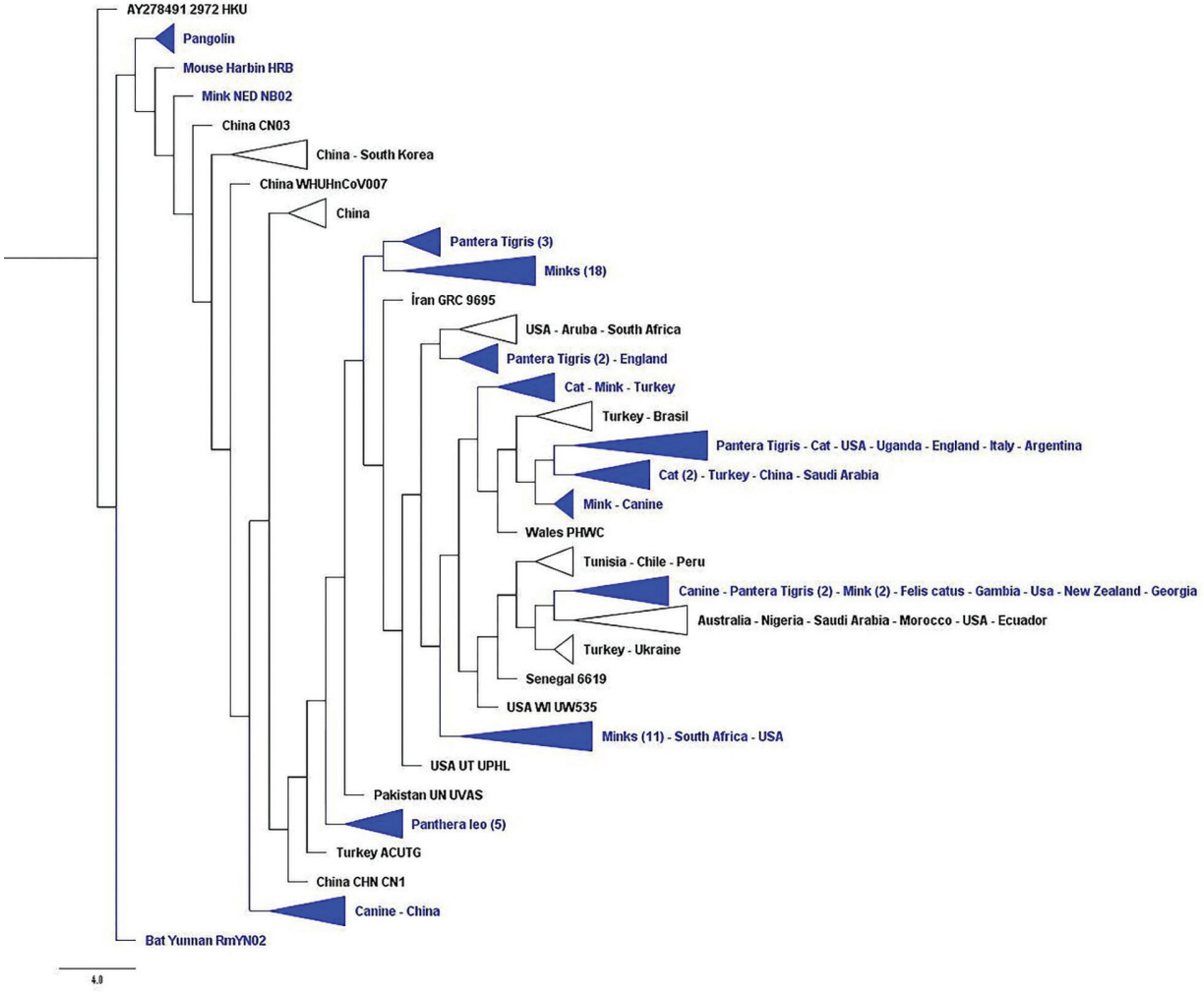

Figure 2. Phylogenetic analysis of full length Spike gene of SARS CoV-2. Blue coloured branches indicated SARS CoV-2 sequences from animals and colourless branches indicated SARS CoV-2 sequences from humans

Nucleotide	Region/ Aminocid number	NC_045512		Mink samples (N:34)			Panthera leo*		Panthera tigris** + Georgia, Australia, New zeland		Human+ Animal***		England ALDP C4812B	
		nuc	aa	nuc	aa	n	nuc	aa	nuc	aa	nuc	aa	nuc	aa
241	-	C		T		26					T		T	
514	-	T		C		8								
913		C											T	
1059	ORF1A/ 265	G	T						T	I				
1380	ORF1A/372	C	A	T	V	11								
1478	ORF1A/405	G	A	A	T	3								
1605-1606-1607	ORF1A/447-448	ATG	ND	XXX	XX	8								
3037	-	C		T		26	C				T		T	
3251	ORF1A/996	G	D	T	Y	4								
3267	ORF1A/1001	C	T										T	I
4206	ORF1A/1314	C	A										T	V
5388	ORF1A/1708	C	A										A	V
5986		C											T	
6954	ORF1A/2230	T	I										C	T
7843		C					T							
8266		C					T							
10829		A		G		5								
11083		G					T							
12795		G		A		13								
13768	ORF1A/4502	A	M				G	V						
14274		G		A		11								
14408	ORF1A/4715	C	P	T	L	26					T	L	T	P
14676		C											T	
14805		C					T							
15279		C											T	
15656	ORF1A/5131	C	T	T	I	15								
15869	ORF1A/5202	A	H				T	L						
16176		T											C	
17247		T					C							
17410	ORF1A/5716	C	R	T	C	8								
17639	ORF1A/5792	C	S				T	L						
18171		C					T							
22344	S/261	G		A	D	4								
22920	S/453	A	Y	T	F	18								
23063	S/501	A	N										T	Y
23064	S/501	A	N	C	T	2								
23075	S/505	T	Y				C	H						

Nucleotide	Region/ Aminocid number	NC_045512		Mink samples (N:34)			Panthera leo*		Panthera tigris** + Georgia, Australia, New zeland		Human+ Animal***		England ALDP C4812B	
		nuc	aa	nuc	aa	n	nuc	aa	nuc	aa	nuc	aa	nuc	aa
23271	S/570	C	A										A	D
23400	S/613	A	Q				G (2)	R (2)						
23403	S/614	A	D	G	G	26					G	G	G	G
23429-23430	S/623	GC					AT AC	I (3) T(2)						
23604	S/681	C	P										A	H
23709	S/716	C	T										T	I
24506	S/982	T	S										G	A
24862		A		G		11[a]								
24914	S/1118	G	D										C	H
25563	ORF3A/ 57	G	Q						T [b]	H				
25936	ORF3A/182	C	H	T	Y	19								
26144	ORF3A/251	G	G				T [c]	V						
27972		C											T	
28048		G											T	
28111		A											G	
28280-81-82	N/3	GAT	D										CTA	L
28881-28882-28883	N/203-204	GGG	RG	AAC[d]	KR	15	GGG				AAC	KR	AAC	KR
28915		C					T							
28977	N/235	C	S										T	F
29685		T		C		5								

Table 1. Nucleotide and amino acid substations in the selected SARS CoV-2 sequences. ([a] southafrica464118; [b] ETLKVET2+ TUR ACUTG-2 + SaudiArabia_ MADINAH1060+ Saudi_Arabia_JEDDAH722; [c] 215193 gb_ MT215193_1_canine_HKG_20-02756_2020_ canine; SouthKorea_KUMC01_2020; [d] gb_MT709105_1_ Felis_catus_FRA_Env-Di_2; gb_MT709104_1_Felis_catus_FRA_Env-Ba_2; gb_MT270814_1_canine_HKG_20-03695_2020; canine_Hong_1_29764_Kong_20_03695_2020_EPI_ISL_450403_2020_03)

Discussion and Conclusion

Due to the rapid circulation of SARS CoV-2 in the world, similar SARS CoV-2 sequences can be found in many countries. Therefore, more data from different species are needed to confirm the origin studies of the SARS CoV-2. The proximity and relationship of SARS CoV-2 circulating between humans and those circulating in animals may also shed light on the future risks of the SARS CoV-2.

Sequences belonging to full genome data of ETLKVET1, ETLKVET2 and ETLKVET3 were generally found to be close to sequences in other countries. The data obtained here also show that the SARS CoV-2 is spreading rapidly to all countries (Adam et al. 2020; Lemieux et al. 2020).

Since there are similar changes in different living species at different times, it seems that SARS CoV-2 can circulate in different species without serious mutation (OIE 2021; El Masry et al. 2020).

When the nucleotide and amino acid sequences of our 3 samples were compared with other SARS CoV-2 samples results, it was observed that there were changes in amino acid sequence of ETLKVET2 (ORF3A/ 57, ORF1A/5809, ORF1A/5584). When looking at its place in phylogenetic trees, it can be evaluated that it is close to the sequences acquired

from Georgia, New Zealand, Australia and Saudi Arabia, Panthera tigris, minks and cats.

When the genetic evaluations on SARS CoV-2 are examined, it is seen that there are many different classifications (Rambaut et al. 2020). Different criteria were evaluated in these classifications and the reliability of the obtained trees was verified with different programs. Since SARS CoV-2 detected in human, canine, mink, leopard, tiger and mouse are found in a single main branch except in bats and pangolins in Wuhan, it is seen that a single genetic type circulates by infecting different species all over the world. It is seen in the data that SARS CoV-2 can circulate in more than one species without serious mutations. However, when the SARS CoV-2 genetic data used in these studies are examined, it is seen that there are small nucleotide and amino acid changes. These nucleotide and amino acid changes (in ORF and Spike proteins) vary by 1-0.5%.

According to the phylogenetic tree data obtained, SARS CoV-2 originates from a branch containing pangolins and RaT13 bat. These findings are consistent with the data described before (Li et al. 2020; Andersen et al. 2020; El Masry et al. 2020).

Although many nucleotide changes are seen in different samples in the genetic sequence of the SARS CoV-2, most of them do not change the amino acid synthesis (Handrick et al. 2020; Gunadi et al. 2020). Therefore, some nucleotide changes may not directly affect the pathogenicity of SARS CoV-2, however, after evaluating where these proteins play a role in SARS CoV-2 with amino acid changes, definite conclusions can be reached.

SARS CoV-2 detections in carnivores in the USA and in people from different countries indicates that they have common mutations. Similar changes seen in these animals in the USA were found in humans in the following months. It is recommended to investigate in which organisms SARS CoV-2 survives in nature, its reservoirs or winter mechanism in the following days.

In addition to England, the sequences (Tygerberg, 464118) obtained from South Africa in April 2020 are similar with the sequences acquired from many minks (in April-November 2020).

SARS CoV-2 detected in Turkey is intertwined with other countries, which shows how fast SARS CoV-2 can spread in the globalizing world. For this reason, countries must act jointly by preparing a common strategy in the fight against human or animal diseases in the future.

This publication reviews SARS CoV-2 genetic data from animals and humans. It has been observed that the genetic data obtained from many animals form a common branch and some of them are located in these branches together with obtained from humans.

In conclusion, it is thought that it will be faster to illuminate the dark points about the spread or treatment of diseases by jointly conducting disease studies in humans and animals within the One Health approach.

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Data Availability: Viral genome sequences are available under Genbank accession numbers MW306666- MW306668; under GISAID accession numbers EPI_ISL_636969- 636971.

Compliance with ethical standards: University of Health Science, Non Interventional Scientific Research Ethics Committee decison number 2020-151

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