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New pieces of the puzzle of the genesis of the SARS-CoV-2 pandemic: A critical review

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Abstract

Knowledge of the underlying process that leads to the generation of a pandemic strain of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is scientifically and politically imperative to identify practices that could generate more devastating pandemics. New evidences are presented here by applying simple descriptive bioinformatics of full genome sequences of human and animal coronaviruses including bat's and pangolin's, BLAST search of the possible origin of the 12-nucleotide insertion in the S1/S2 cleavage site, and a map of the presence of accessory proteins of SARS-CoV-2 in those coronaviruses. Together with published wide coverage of SARS-CoV-2 and other coronaviruses, we reconstruct the probable underlying process of pandemic strain generation. Based on overwhelming data, the trade and farming of animals that harbors SARS-CoV-2-like viruses should be stopped immediately.

Keywords: SARS-CoV-2; Origin; New evidence; Pandemic generation

1 Introduction

Understanding the origin of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the underlying process that led to the generation of the pandemic strain is of high global scientific and political interest to identify practices that could generate the next more devastating pandemic. With reference to the Spanish flu pandemic that had a death toll of approximately 50 million [1], roughly 3% of the world population at that time, a pandemic that is more severe than the ongoing coronavirus disease 19 (COVID-19) might occur in the future. The fatality could reach more than 200 million of a total global population of eight billion. Even with a much lower death toll of six million as of April 14, 2022 (https://covid19.who.int/), the impact of this modern COVID-19 pandemic has been very devastating. The health system was overwhelmed. A great amount of expenditure must be allocated to curb its impact, which goes far beyond the health system. The current pandemic severely affects the global economy, social life and livelihood [2, 3].

A large body of literature has covered the origin of SARS-CoV-2. It is generally believed that the catalyst of the pandemic was a zoonotic event [4-6]. Accumulated evidence indicated the epicenter of the pandemic as the Huanan Seafood Market in Wuhan, China. At the very early stage of the pandemic, the majority of cases were linked to the market [7]. Convincing evidence came recently in the form of a preprint article that stated that the virus was traced to that market and the surrounding environment at the start of January 2020 [8]. Using spatial analyses and studying the origin of genomic diversity, researchers, as shown in a pre-published paper, are convinced that the emergence of SARS-CoV-2 occurred via the live wildlife trade and that the Huanan market was the unambiguous epicenter of the COVID-19 pandemic [9, 10]. Previously, a joint report of the World Health Organization concluded that the steep increase in mortality occurred in the beginning of January 2020 among the population in Hubei Province outside Wuhan [11]. While a review of influenza-like illness cases in Wuhan city in October and November 2019 indicated that any substantial

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transmission of SARS-CoV-2 infection in Wuhan before late December 2019 was considered unlikely [11]. All showed that the epidemic had just begun.

However, the direct ancestor and the process leading to the generation of the pandemic strain have yet to be discovered. Information on the origin and intermediate hosts is also missing. Previously, it was suggested that the virus originated from bats [12, 13]. Another group claimed the pangolin to be its probable origin [14]. Contradictorily, those animals have not been sold in the Huanan Market since at least May 2017 [15]. Therefore, the notion that the market was the epicenter of the pandemic is unjustified.

Here, author critically revisited the issue of the origin of SARS-CoV-2 and provides new evidences to reconstruct the events that led to the genesis of the pandemic strain.

2 Coronavirus genome

To draw a comprehensive understanding of the molecular events that led to the emergence of the pandemic SARS-CoV-2 strain, the genome organization of SARS-CoV-2 and other coronaviruses should be referred to. SARS-CoV-2 belongs to the genus *Betacoronavirus* of the family **Coronaviridae**, which is characterized by a large genome of approximately 30 kb of positive-sense single-stranded RNA [16, 17]. Its RNA is linear, unimolecular, infectious, capped, polyadenylated and structurally polycistronic [18]. The genus includes human viruses, i.e., HCoV-OC43, HCoV-HKU1, SARS-CoV-1, MERS-CoV, and SARS-CoV-2, and animal viruses, i.e., various bat and pangolin coronaviruses [19].

The genomes of various coronaviruses have slightly different organization. All coronavirus genome codes for structural proteins spike (S), matrix (M), envelope (E) and nucleoprotein (N), a nonstructural protein of a large RNA-dependent RNA-polymerase complex annotated as open reading frame 1AB (ORF1AB), as well as various accessory proteins [20]. Structural proteins and polymerase complexes occur in all family members. The number of accessory proteins varies, even within a certain genus. With additional 5' and 3' UTR and intergenic sequences (IGSs), the genome organization of SARS-CoV-2 is 5'-UTR-ORF1AB-IGS-Spike-IGS-ORF3A-ORF3B-IGS-Protein E-IGS-membrane (MA)-IGS-ORF6-ORF7A-ORF7B-ORF8-IGS-Nucleoprotein NP-ORF10-3'-UTR [21, 22]. The IGSs have been identified previously [20, 23, 24]. The IGS, also known as transcription-regulating sequence (TRS), of coronaviruses has a consensus sequence of CUAAAC or UAAACGAAC, which may influence translation rate [25, 26]. Database wide survey on the pattern of IGS in SARS-CoV-2 needs to be conducted.

In resolving the origin of SARS-CoV-2, we should widen our focus to the whole genome as well as its organization. Most reviews thus far have mainly focused on the spike protein which is reasonable since the protein is a major pathogenic coronavirus determinant. This surface protein possesses major immunogenic domains, and most gene-based vaccines target only the spike gene of SARS-CoV-2. The protein is highly glycosylated and cysteine rich, with two cleavage sites: S1/S2 and S2′ [27]. The glycosylation pattern of the SARS-CoV-2 spike involves N-linked and O-linked glycosylation [28]. The spike protein also has two protease cleavage sites, which are critical for virus activation and replication as well as a critical determinant of coronavirus tropism and pathogenesis [29]. The S1 domain, which carries major antigenic determinants, mediates receptor recognition and viral attachment to initiate host cell entry [30]. The N-terminal domain (NTD) contributes to host range [31]. Binding of the receptor-binding domain to receptors initiates infection [32, 33], and the S2 domain mediates membrane fusion [30, 34, 35].

3 On the origin

Previously, the Bat_RaTG13 and other bat coronaviruses are thought to be the closest relative of SARS-CoV-2, which have "the highest average genetic similarity to SARS-CoV-2, although it is not is not the progenitor of SARS-CoV-2" [4]. More recently bat coronaviruses related to SARS-CoV-2 have been identified in Laos [36]. It is proposed that the backbone of SARS-CoV-2 is a Bat_BANAL-20-52/Laos/2020-like virus as described below.

Alignment of full genome sequences available in GenBank of SARS-CoV-2 strain of Wuhan-Hu-1 with 149 other human, bat, pangolin, and other animal coronaviruses, as available upon request to the author, shows that the full genome of SARS-CoV-2 is almost perfectly aligned with bat Bat_BANAL-20-52/Laos/2020 and Bat_RaTG13, not with other bat and pangolin coronaviruses. Print screen of whole genome alignment of Wuhan-Hu-1, Bat_BANAL-20-52/Laos/2020, Bat_RaTG13 and the closest pangolin coronavirus of Pangolin_MP789 is shown in Figure 1. The illustration demonstrates that extensive genome gaps occurred in pangolin_MP789 virus. Based on the genome gaps, Wuhan-Hu-1 is closer to Bat_RaTG13 than to Bat_BANAL-20-52/Laos/2020. However, number of nucleotide difference and genetic distance as depicted in Table 1 show otherwise, that Wuhan-Hu-1 is closer to Bat_BANAL-20-52/Laos/2020 than to

Bat_RaTG13, as the Pangolin_MP789 is distancing much further. Other Laos bat coronaviruses annotated as Bat_BANAL-20-xxx are distancing a bit further than the Bat_BANAL-20-52/Laos/2020 strain (not shown).

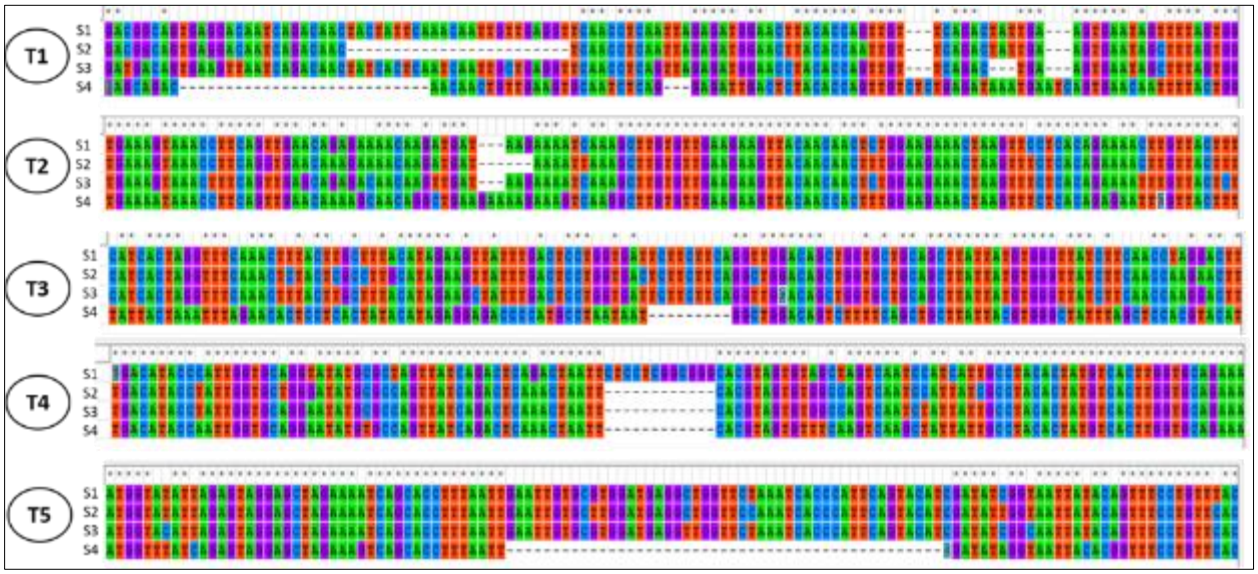

Figure 1 Print screen of alignment of Wuhan-Hu-1 (S1), Bat_BANAL-20-52/Laos/2020 (S2). Bat_RaTG13 (S3), and Pangolin_MP789 (S4) coronaviruses. Only some windows are shown that demonstrating extensive gaps are shown. Track 1 (T1) is sequences between nucleotide (nt) number 3200-3250; Track 2 (T2) is sequences between nt 3900-4000; Track 3 (T3) is sequences between nt 22250-22350; Track 4 (T4) is sequences between nt 23550-23650, around the S1/S2 cleavage site unique to Wuhan-Hu-1. Track 5 (T5) is sequences between nt 28000-29000

Additionally, as shown in Table 1, 749 transitions and 162 transversions occurred between Wuhan-Hu-1 and Bat_BANAL-20-52/Laos/2020, while the numbers were 940 and 194 to bat_RATG13, and 1944 and 922 to Pangolin_MP789. R value, which is the number of transition (S) divided by the number of transversions (V), between Wuhan-Hu-1 and Bat_BANAL-20-52/Laos/2020 is 4.93, while to Bat-RaTG13 and Pangolin_MP789 were 4.85 and 2.11, respectively. This simply means that transition is the most frequent in Wuhan-Hu-1 and Bat_BANAL-20-52/Laos/2020 comparison than Wuhan-Hu-1-Bat-RaTG13 and Pangolin_MP789.

To demonstrate the phylogenetic relatedness between SARS-CoV-2 virus of Wuhan-Hu-1 strain to Bat_BANAL-20-52/Laos/2020, Bat_RaTG13, and Pangoline_MP789, phylogeny of the aligned full genome sequence of the viruses was conducted by using the Maximum Likelihood method and Kimura 2-parameter model in Mega11 [37]. The phylogenetic tree is shown in Figure 2. The tree is again demonstrating that Wuhan-Hu-1 strain is closely related to Bat_BANAL-20-52/Laos/2020 with bootstrap value of 100%.

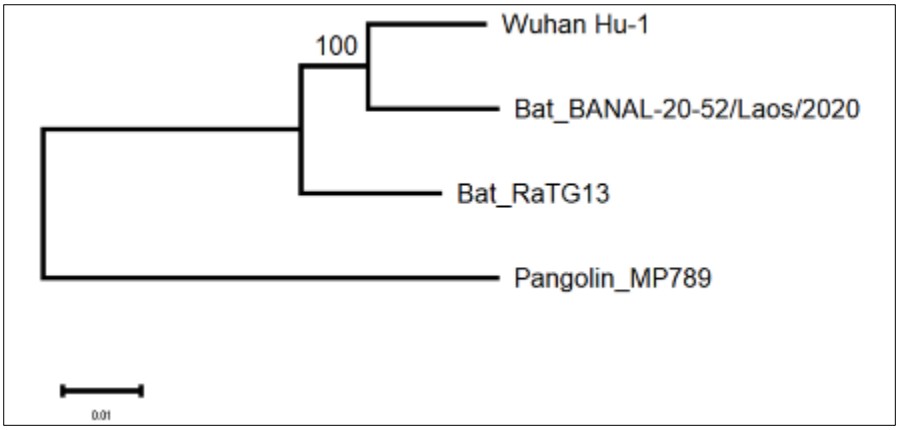

Figure 2 Phylogenetic reconstruction of the full genome of SARS-CoV-2 strain of Wuhan Hu-1 with selected bat and pangolin coronaviruses. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model in Mega11 [**37**]

Table 1 Genetic distances, number of transitions and transversions of nucleotide difference of Bat_BANAL-20-52/Laos/2020, bat-RATG13, Pangolin_MP789 to SARS-CoV-2 of Wuhan-Hu-1 strain

Virus	Genetic distance to Wuhan-Hu-1	Number of transitions of the polymorphic nucleotides to Wuhan-Hu-1 (S)	Number of transversions of the polymorphic nucleotides to Wuhan-Hu-1 (V)	R=S/V
Bat_BANAL-20-52/Laos/2020	0.0312	749	152	4.93
Bat-RATG13	0.0395	940	194	4.85
Pangolin_MP789	0.1051	1944	922	2.11

Alignment and phylogenetic analysis of Wuhan-Hu-1 sequence with 149 human and animal coronavirus full genome sequences available in GenBank were conducted using Clustal Omega available online at EMBL-EBI (www.ebi.ac.uk). The closest sequences to Wuhan-Hu-1 were selected and further analysis in Mega11 [37] to calculate genetic distance, number of transitions and transversions as well as R=(S/V) values. Genetic distance was calculated using Kimura-2 parameter.

4 New pieces of puzzle

The donor of the 12-nt insertion in the cleavage site of the spike protein is key to unravelling the mystery of the generation of the pandemic strain. We conducted a BLAST search of the 12-nt insertion with an additional two nucleotides at both ends and focused our search on the mRNA or transcriptome, as the coronavirus is a positive-strand RNA virus that replicates in the cytoplasm. The search string of TT**CTCCTCGGCGGG**CA, in which the 12-nt insertion is bolded and underlined, resulted in 100% query cover and percentage identity of the bat *Myotis brandtii* AT-rich interactive domain 4B and *Molossus molossus* coiled-coil glutamate-rich protein 2 mRNAs. However, the geographical ranges of *Myotis brandtii and Molossus molossus* do not fit the genesis of the pandemic SARS-CoV-2 strain. *Myotis brandtii* occurs in central and northern Europe, while *Molossus molossus* occurs in North to South America as well as the Caribbean. Moreover, a scientific report claimed that no bat had been sold at the Huanan Seafood Market since 2017 [15].

Even though the geographical range of those bats does not fit and bats were not traded in Huanan, bats should be listed as probable donors of that 12-nt insertion. The transcriptome or even full genome of all bat species are not available in GenBank. Virus spillover could have occurred from wild and free-roaming bats. The mystery bat must have a geographical range in Asia. A shorter oligonucleotide blast of 5'-T**CTCCTCGGCGGG**CA-3' resulted in the mRNA or transcriptome of the *Rhinolophus ferrumequinum* bat, which occurs in North Africa and southern Europe through Southwest Asia, the Caucasus, Iran, Afghanistan, Pakistan and the Himalayas to Southeastern China, Korea, and Japan (https://www.eurobats.org); *Pteropus alecto*, which is native to Asia and Australia (https://animaldiversity.org); *Hipposideros armiger,* which has a geographical range of South Asia, Southeast Asia, and China (http://www.bio.bris.ac.uk); *Pipistrellus kuhlii,* which extends from the Mediterranean area, expanding to the North, over the whole Arabian Peninsula to Kazakhstan and Pakistan (https://www.eurobats.org); *Pteropus giganteus,* which is distributed across the Indian subcontinent, including Bangladesh, Bhutan, India, Tibet, Maldives, Myanmar, Nepal, Pakistan and Sri Lanka (https://animaldiversity.org); and *Myotis myotis,* which occurs in Western, Central and Southern Europe, as well as in Asia Minor (https://animaldiversity.org).

The blast of the TT**CTCCTCGGCGGG**CA search string also resulted in human, mammal, and avian mRNAs. The blast with pangolins taxa resulted in the highest query cover of only 87% with pangolin mRNAs. Thereafter, humans and mammals are also potential donors, while birds can be the least possible, as birds seem unsusceptible to SARS-CoV-2 infection [38, 39]. Many wild mammals were traded in Huanan [15]. Free-roaming cats were very likely present in the market. Cat samples, in Wuhan and not in Huanan Market, were reported to be serologically positive shortly after the discovery of SARS-CoV-2 [40]. Author presumes that the direct ancestor of SARS-CoV-2 seemed to have been circulating unnoticed in the Huanan Market, as it was traced to that market and the surrounding environment in early January 2020 [8]. The hypothesis of cat as the donor is supported by evidences that infection of SARS-CoV-2 strains following infection of cats, among others, has been reported to be resulted in rapid strain selection [41]. This animal has been proven to be well adapted to SARS-CoV-2 too [39]. Meanwhile, a human donor is hard to explain. The adaptation of an animal virus into a human pathogen is rarely discussed. This is not impossible, though. Theoretically, initial minimum and subclinical infection (in the human body) can lead to mutation and recombination of the agent that leads to selection of a descendant virus that fits the human body.

Since accessory proteins have been reported to have significant roles in pathogenesis and human adaptation of SARS-CoV-2 [42], we analysed the ORFs of all accessory proteins of SARS-CoV-2 in human and animal coronaviruses. The problem is that the annotation of accessory proteins of various coronaviruses is confusing based on data entry in GenBank. The annotation in the GenBank database was not unified. To solve the problem, we aligned each accessory protein of Wuhan-Hu-1 separately to determine whether each ORF was available in the genomes of various human and animal, including bat and pangolin coronaviruses. The result is presented in Table 2. All six accessory protein ORFs are not present in bovine, feline, canine, and avian coronaviruses (not shown). Interestingly, all six accessory proteins of SARS-CoV-2 are available in bat and pangolin coronaviruses with some variations in length. SARS-CoV shares ORF6, ORF7A, and ORF10 with SARS-CoV-2, while ORF3A, ORF7B, and ORF8 are missing. The nature of SARS-CoV infection is limited human-to-human infection, multiorgan involvement in fatal cases and a fatality rate of approximately 9% [43]. None of the accessory proteins of SARS-CoV-2 exist in Middle East respiratory syndrome (MERS-CoV), human 229E, human OC43, or human HKU1. The MERS-CoV was resembling SARS-CoV in transmissibility and clinical signs, except for a much higher fatality rate of more than 30% [44].

Table 2 Differences of accessory proteins coding region assignment of the whole genome of various coronavirus in human, bat, and pangolin to SARS-CoV-2

	Open Reading Frame (ORF)[1]					
Virus	**ORF3A**	**ORF6**	**ORF7A**	**ORF7B**	**ORF8**	**ORF10**
Human SARS-CoV	Absent	Present	Present	Absent	Absent	Present
Human MERS-CoV	Absent	Absent	Absent	Absent	Absent	Absent
Human 229E	Absent	Absent	Absent	Absent	Absent	Absent
Human OC43	Absent	Absent	Absent	Absent	Absent	Absent
Human HKU4/P251T	Absent	Absent	Absent	Absent	Absent	Absent
Bat RaTG13	Present	Present	Present	Present	Present	Present
Bat SL-CoVZXC21	Present	Present	Present	Present	Present	Present
Bat_BANAL-20-52/Laos/2020	Present	Present	Present	Present	Present	Present
Bat_BANAL-20-103/Laos/2020	Present	Present	Present	Present	Present	Present
Bat_BANAL-20-236/Laos/2020	Present	Present	Present	Present	Present	Present
Bat_BANAL-20-247/Laos/2020	Present	Present	Present	Present	Present	Present
Pangolin GX_P2V	Present	Present	Present	Present	Present	Present
Pangolin MP789	Present	Present	Present	Present	Deleted 16 residues[4]	Present
Pangolin PCoV_GX-P3B	Present	Present	Present with uncertain amino acids[2]	Present	Present	Present
Pangolin GX-P2V	Present	Present	Present	Present	Present	Present
Pangolin GX-P5E	Present	Present	Present	Truncated 26 residues[3]	Present	Present
Pangolin CoV_GX-P5L	Present	Present	Present	Present	Present	Present
Pangolin CoV_GX-P1E	Present	Present	Present	Present	Present	Present

[1]Based on sequence annotation ofSARS-CoV-2 strain of Wuhan-Hu-1 (Accession Number NC_045512); [2]Long track of un-definitive (N) available in the ORF; [3]Truncated due to present of stop codon because of 4-nt deletion; [4]deletion of 16 amino acids

The accessory proteins of concern are proposed to be ORF3A, ORF7A, ORF7B, and ORF8. Those proteins are the highest percentages of polymorphic amino acids between Bat_BANAL-20-52/Laos/2020 to Wuhan-Hu-1 (Table 3). Since ORF7A is present in SARS-CoV, this protein might not contribute in human adaptation. ORF6 comparison resulted in 1.6% different residues. The ORF10 is even homolog between the strains.

Table 3 Genetic distance and number of polymorphic amino acids of accessory proteins of the Bat_BANAL-20-52/Laos/2020 to Wuhan-Hu-1

Accessory Protein Gene	Length of the protein (amino acids)	Genetic distance to Wuhan-Hu-1	Number of amino acid difference (% from the length)
ORF3A	275	0.0468	12 (4.36%)
ORF6	61	0.0332	1 (1.6%)
ORF7A	121	0.0396	4 (3.31%)
ORF7B	43	0.0231	3 (6.98%)
ORF8	121	0.0366	5 (4.13%)
ORF10	38	0.000	0 (0.00%)

Genetic distance was calculated using Kimura-2 parameter in Mega11 software [37].

5 Discussion

To date, unless a closer virus will be discovered, the direct progenitor of SARS-CoV-2 should have been the ancestor of the Bat_BANAL-20-52/Laos/2020-like virus. Since the Bat_BANAL-20-52/Laos/2020 has only been identified in 2020, the yet-unknown ancestor must have generated SARS-CoV-2, the Bat_BANAL-20-52/Laos/2020, on many other offspring. The main underlaying mechanisms are most likely simple mutations and recombination of mostly short nucleotide except for S1/S2 cleavage site. A theory of extensive recombination as proposed recently [36] might not be likely as there is no genetic pool of bat coronaviruses discovered in one place in one species.

As shown before, if we propose that SARS-CoV-2 emerged from Bat_BANAL-20-52/Laos/2020, Bat_RaTG13, or Pangolin_MP789, transition mutations must have occurred more frequently than transversions. The R values of from Bat_BANAL-20-52/Laos/2020, and bat_RATG13 if evolved to generate SARS-CoV-2 were close to five, which means number of transitions was five times higher than transversions. If the pangolin_MP789-like were the ancestor, it must have needed more extensive transversions.

The transitions are indeed more frequent than transversions in viral mutations [45]. The gaps must have been occurred trough recombination. Mutation and recombination are common mechanisms of viral diversity in coronaviruses. Although coronaviruses possess an RNA polymerase that has a 3′-exonuclease domain [46], the mutation rate in the SARS-CoV genome was estimated to be equal to that in other RNA viruses [47]. In addition, coronavirus RNA exists naturally in secondary structure forms, which promote template switching and produce recombination [48]. Recombination is indeed a shared machinery of the genetic diversity of coronaviruses in gaining tissue tropism and virulence in addition to genetic mutation [49].

Regarding influenza viruses, we learned that for a pandemic to emerge, the causative agent must occur suddenly and have a completely new antigenic structure for which the human population does not have immunity [50]. This should have been valid in the emergence of the ongoing COVID-19 pandemic.

To reconstruct a plausible molecular event that leads to pandemic strain generation, the question is what gene(s) or gene fragments can be associated with the capability to trigger the current pandemic. In this regard, the spike protein is not a strong candidate. The receptor binding site (RBS) in this protein and receptor binding capacity to angiotensin-converting Enzyme 2 *(ACE2), t*he receptor of SARS-CoV-2 [51], are not unique to SARS-CoV-2. Various animals have been reported to be susceptible to SARS-CoV-2 in natural and experimental infections. In fact, other human coronaviruses also use the same receptor of ACE2; some have a clinical course of severe disease, such as SARS-CoV, but limited human-to-human transmission [43], while others, such as human 229E, human OC43, and human HKU1, are easily transmissible but cause self-limiting clinical consequences [52].

A non-stringent selection in receptor recognition is indicative of various animal natural and experimental infections. Beside cats as described above, efficient SARS-CoV-2 replication, which includes receptor recognition, occurs in fruit bat and mink. An experimental infection of SARS-CoV-2 in fruit bats produced systemic infection without clinical signs [38]. Another proof of the non-stringent receptor recognition is the high susceptibility of mink to SARS-CoV-2 infection. Since April 2020, outbreaks of SARS-CoV-2 on mink farms were reported in the Netherlands, Denmark, United States, France and many others [53]. In a lab experiment, the receptor activity of ACE2 from 14 mammal species has been found

to support the infectious entry of lentiviral particles pseudotyped with the wild-type or furin cleavage site-deficient S protein of SARS-CoV-2 [54].

The polybasic cleavage site does not fit to be a determinant of human adaptation in SARS-CoV-2, either. One molecular hallmark of SARS-CoV-2 is the presence of PRRA motifs and O-linked glycosylation in the S1/S2 cleavage site [55, 56]. The 12-nt insertion at the S1/S2 cleavage site generates PRRA motifs and seems not to be the last event that leads to epidemics and pandemics. The PRRA motif was very stable in SARS-CoV-2 until the emergence of variants that become RRRA motifs in Delta or HRRA motifs in Alpha, Omicron, and GH/490 variants/subvariants [57]. The PRRA motif might be responsible for the multiorgan involvement of SARS-CoV-2-infected patients and modulates cellular tropism and ACE2 usage [58], but not accountable for human adaptation. Bat-like SARS-CoV-2 without a PRRA motif exists naturally and remains transmissible in human [59]. The multiorgan involvement capacity is facilitated by ubiquitous presence of the protease for the cleavage of the PRRA motif. The polybasic cleavage site of haemagglutinin of influenza virus has been established as a determinant of highly pathogenic avian influenza, which is characterized by multiorgan infection, not human adaptation [60-62]. The Delta variant has been reported to have increased toxicity of the S protein and pathogenicity compared to Wuhan-Hu-1 [63]. The HRRA motif in Omicron might need a different class of proteases to cleave the spike protein of SARS-CoV-2, which is mainly present in the upper respiratory tract, causing less severe infection than Delta, as has been reported [57, 64].

Moreover, other coronaviruses with monobasic cleavage sites can be transmitted between humans. Again, this fact supports the statement that a polybasic cleavage site is not a determinant of human-to-human transmission. The PRRA motif also occurred in MERS-CoV but not in SARS-CoV. Both strains had the capacity of human-to-human transmission in hospital settings, not community settings [43, 44]. MERS-CoV has a CFR of more than 30% [44], while SARS-CoV has a CFR of approximately 9% [43]. However, the CFR of SARS-CoV-2 is much less than that of both strains.

Interplay between viral proteins and host genetic factors seem to lead to the overall biological characteristics of coronaviruses. An interaction with host genetic factors to produce severe clinical disease in SARS-CoV-2 only came to light recently. Scientists identified a host of genetic variants that are linked to an increased risk of developing severe clinical signs of SARS-CoV-2 infection [65]. There are two distinct mechanisms that predispose patients to life-threatening disease, i. e. failure to control viral replication and an enhanced tendency towards pulmonary inflammation and intravascular coagulation [65].

Acquisition of human adaptation could be “the last supper” or the last critical genetic changes of the presumably ancestor of the Bat_BANAL-20-52/Laos/2020-like virus to be able to trigger pandemic. Four accessory proteins of ORF3A, ORF7A, ORF7B, and ORF8 are strong candidates for human adaptation of SARS-CoV-2, which have polymorphic residues of 4.36%, 3.31%, 6,98%, and 4.13% between Wuhan-Hu-1 and the Bat_BANAL-20-52/Laos/2020, respectively. The mutations of these proteins could be the last decisive changes prior to the birth of the pandemic strain. Except for ORF7A, the other these ORF’s were missing in SARS-CoV (Table 2), which has been recognized to have a limited human-to-human transmission [43], is an indirect evidence of the role of those ORF’s in human adaptation, hence in pandemic generation. Moreover, the role ORF8 in SARS-CoV-2 adaptation to humans has been reported convincingly [42, 66, 67]. Although less diverged, the other two accessory proteins are not less important. SARS-CoV-2 ORF3A is a viroporin that disrupts cell integrity through endocytosis, endomembrane-associated replication, and viral release through exocytosis [68]. This protein is also a proinflammatory immune response inducer that can trigger a cytokine storm, especially under hypoxic conditions. It induces cell death through apoptosis, necrosis, and pyroptosis [68]. ORF7B contributes to pathogenesis through tumor necrosis factor-α-induced apoptosis in cells [69].

Many animals and people could be the host of the final adaptation in Huanan. Special attention is to be given to cats and bats. The susceptibility of cats has been described above. If bats were absent among the animals sold long before the pandemic started, wild bats might have been roaming in the market and cause virus spillover. We are not certain on the present of free roaming bats in Huanan Seafood Market, however, we understand that markets in Asia usually provide various species of wild and domesticated animals; some are free roaming, such as dogs, cats, rats, bats, sparrows, and swiftlets, and crowds of people allow the transmission from animal to animal as well as from animal to human and vice versa. The animal welfare of traded animals is typically compromised and the animals are usually kept in narrow cages with minimal food. In this situation, animals are under stress and severely immunocompromised, which allows any agent to change drastically. This has even been discovered in SARS-CoV-2 itself [70, 71].

Important clues are missing from Huanan-Wuhan. The serological data should have been collected easily from all animals in the market as well as from people working in the market immediately in early January 2020. Samples from Huanan workers before December 2019 should have been traceable and detectable. It would be an odd occurrence and invite suspicion if there were never a plan to collect and analyze such data.

The probable event that led to the generation of pandemic SARS-CoV-2 was then reconstructed, as shown in Figure 3. The proposed ancestor is the progenitor of the Bat_BANAL-20-52/Laos/2020. The ancestor underwent gradual mutation and/or recommendation since before 2019 generating many offsprings, including SARS-CoV-2, Bat_BANAL-20-52/Laos/2020, and many others. The ancestor was transmitted between bats or bats to other animals, including cat, or to human. The 12-nt insertion that generated a PRRA motif in the S1/S2 cleavage site might have occurred before the end of 2019. This direct ancestor might have spread with no capacity for human adaptation. Spill over from the ancestor to humans or mammals in the Huanan Seafood Market at the end of 2019, led to silent infection of the strain until it gained final substitutions of viral proteins ORF3A, ORF7A, ORF7B, and/or ORF8, responsible for human adaptation in Huanan Seafood Market. The 12-nt insertion could have been occurred in that market too. In the late December 2019, animal to human or human to human transmission occurred in that Market, until the first human case was confirmed in Wuhan, China. The WHO declared this novel coronavirus infection, later known as SARS-CoV-2, as public health emergency of international concern on January 30, 2020. On March 11, 2020, the organization declared COVID-19 pandemic

The author is fully aware that he has been very speculative throughout this manuscript. The author does not have direct evidence at all. Instead of challenging our opinion, we should presume it is true. Moreover, alarming facts are clear in the alignment of the accessory ORF of Wuhan-Hu-1 (Table 2). All bat and pangolin strains already possess all ORFs that SARS-CoV-2 owns. Thereafter, new human pathogenic strains can be "in the making". These strains might need some changes to be able to infect humans and trigger another disaster. The trade and farming of animals, as well as the collection of a large number of wild animals which come into close contact with domesticated animals and crowds of people, should be stopped immediately; otherwise, we may experience another health, social, and economic disaster again in the near future.

As it is clear that various accessory proteins, especially but not limited to ORF3A, ORF7B, and ORF8, are crucial in human adaptation, the next vaccine should be prepared based on those proteins to prevent clinical signs more effectively, regardless of which SARS-CoV-2-like virus emerges.

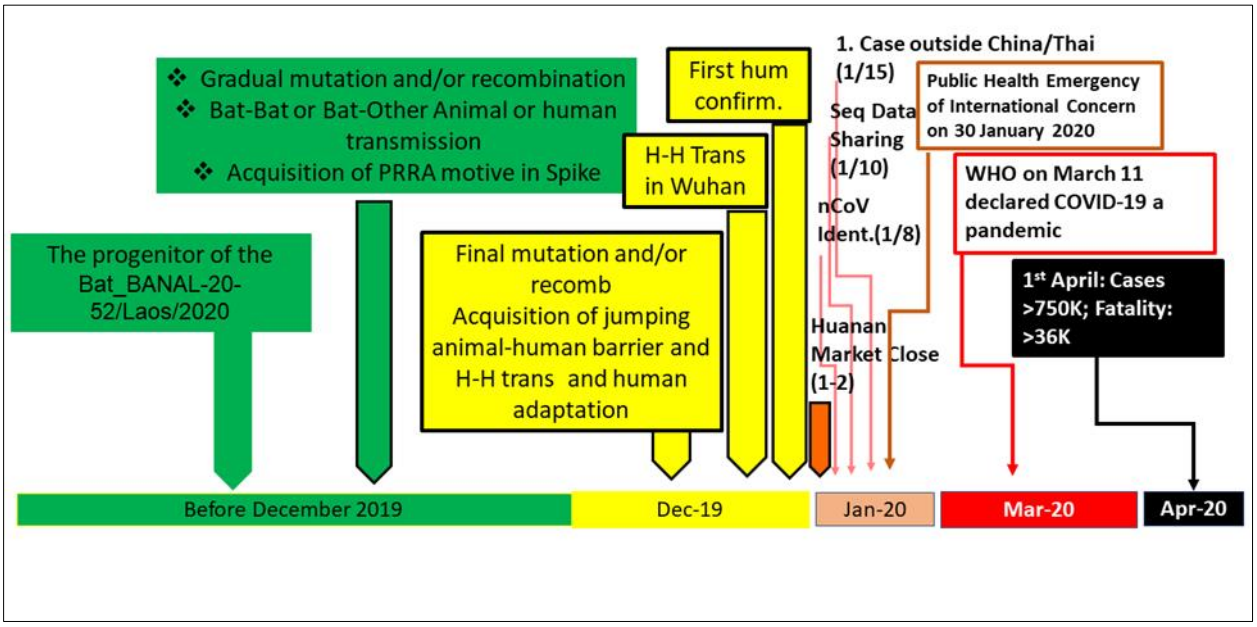

Figure 3 The probable event that led to the generation of pandemic SARS-CoV-2. The proposed ancestor underwent gradual mutation and/or recommendation generating many offspring including SARS-CoV-2, Bat_BANAL-20-52/Laos/2020, and many others. The ancestor was transmitted between bats or bats to other animals, including cat, or to human. The 12-nt insertion that generated a PRRA motif in the S1/S2 cleavage site might have occurred before the end of 2019. The WHO declared this novel coronavirus infection, later known as SARS-CoV-2, as public health emergency of international concern on January 30, 2020. On March 11, 2020, the organization declared COVID-19 pandemic

6 Conclusion

In conclusion, mutation and recombination were the driving forces in the generation of the pandemic SARS-CoV-2 strain. Recombination with the mRNA of bats or mammals, especially but not limited to cats and humans, resulted in a 12-nt insertion in the S1/S2 cleavage site of the spike protein. The SARS-CoV-2 accessory proteins ORF3A, ORF7B, and ORF8 are missing in SARS-CoV, which could lead to better human adaptation of SARS-CoV-2 than SARS-CoV. The trade and farming of animals that harbor SARS-CoV-2-like viruses should be stopped immediately.

Compliance with ethical standards

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Disclosure of conflict of interest

Author claims no conflict of interest.

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Data Availability

A fasta file for the generation of the dataset is available upon request.

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