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Perspective

Lacunae in the natural origin of severe acute respiratory syndrome coronavirus 2

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Abstract: The exact origin of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) responsible for unleashing the pandemic Corona Virus Disease (Covid-19) is still not established unambiguously. The intermediate and reservoir hosts of SARS-CoV-2 needs to be identified with clarity and how the disease exploded into a pandemic, inevitability need urgent scientific answers to contain and prevent future pandemics and crises. This perspective provides awareness of the peculiar features of SARS-CoV-2 and inspects the gaps in the natural zoonotic origin of the pandemic.

Keywords: chimeras; coronavirus; Covid-19; mutation; origin; SARS-CoV-2; zoonosis

Introduction

The pandemic known as Corona Virus Disease (Covid-19) caused by the Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) has resulted in a major global disaster claiming millions of lives, crippling health and economy throughout the world. Earlier episodes of epidemics caused by Coronavirus (CoV), though of less severity, in 2002 called the severe acute respiratory syndrome (SARS-1) and later in 2012 called the Middle East Respiratory Syndrome (MERS) had bats as their reservoir hosts [1]. The intermediate hosts for SARS-1 were the civet cats while that of MERS was identified to be dromedary camels [2]. What actually caused the outbreak and spread of the current pandemic warrants a scientific investigation in order to prevent and contain the future spread of infectious diseases. This paper provides insights into the various aspects regarding the origin of the pandemic and the peculiarities of SARS-CoV-2 with respect to other coronaviruses.

Taxonomy, classification and structure

Coronaviruses (CoVs), single-stranded RNA viruses belonging to the Coronaviridae family, are divided into 4 genera based on their genetic sequences - Alpha-CoV, Beta-CoV, Gamma-CoV and Delta-CoV. These genera of CoVs are known to harbor in diverse species of horses, cattle, pigs, cats, birds, rats, ferrets, rabbits and many wild animals [3].

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The Gamma-CoV have been isolated from whales and birds while Delta-CoV mainly resides in the pigs and birds. But many of these CoVs are not infective in man. However, the Alpha-CoV and Beta-CoV can infect rodents and humans [4]. There are seven human infecting CoVs (HCoVs) known till now. These are HCoV-229E and HCoV-NL63 (both belong to Alpha-CoVs), HCoV-OC43, HCoV-HKU1, SARS-CoV-1, MERS and SARS-CoV-2 (all 5 belong to Beta-CoVs group). Infection with HCoV-229E, HCoV-OC43, HCoV-HKU1 and HCoV-NL63 can show symptoms of mild or severe cold along with or without diarrhea [5]. Meanwhile, SARS-like respiratory infection along with severe acute respiratory distress and many extrapulmonary manifestations are accompanied by infection caused by SARS-1, MES and the most recent SARS-CoV-2 [4].

The beta-CoV genus, which has most of the HCoVs is again subdivided into four lineages - A, B, C and D [4]. Any novel isolate of CoV having pathogenic potential would be designated as virus /host/location/isolate/date (for example; SARS-CoV-2/human/Wuhan/X1/2019) as proposed by the Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. In publications, this would be extended along with the sequence database ID associated with the specific virus genome in a public database such as GenBank [6].

The CoVs have the largest RNA genomes of all the RNA viruses. The 28-32kb positive single-stranded RNA sequences of the CoV has different structural genes such as H (coding for Hemaglutinin, absent in SARS-CoV), S (coding for Spike), E (coding for Envelope), M (coding for Membrane) and N (coding for Nucleocapsid) at its 3'end whereas the replicase locus is coded in the 5'end [7]. Under an electron microscope, the spike proteins protruding on the surface of the envelope proteins provide the CoVs a crown-like morphology, hence the name 'corona' [8]. The Spike

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(S) glycoprotein has two domains - S1 (forming the bulb region of the spike) and S2 (the highly coiled stalk portion) when cleaved by a trypsin-like host serine protease. The S2 domain is conserved in CoVs and is capable of conformational alterations enabling viral entry into the host cell. S1 domain is divergent in CoVs and binds to host target receptors. This binding leads to conformational changes in the highly conserved S2 domain of the spike resulting in membrane fusion between E of the virus and host cell. The species range and tissue tropism of the virus are determined by the interaction of S1 and host cell receptors. For successful viral entry into host cells, the S1 and S2 break apart followed by internalization of the virus into the host cell by receptor-mediated endocytosis [7].

Zoonotic origin

The transmission of a pathogen from wild animals to humans is termed zoonosis. The main factors in a zoonotic disease are the pathogen which can be a bacterium, protozoan or virus (SARS-CoV-2 in case of Covid-19) and the host. The reservoir host (also known as natural, ancestral or maintenance host) is one in which the infectious agents' habitat for years or decades, without usually being pathogenic, is in a decent, stable and long-term relationship. Bats are considered as the reservoir hosts for CoVs and the viruses cannot disappear or reduce from their permanent dwelling reservoirs, the bats. The intermediate host serves as a transient or amplifier host which becomes infected by the CoVs before or while getting introduced to the human population. The virus multiplies in the intermediate host which acts as a source for the zoonotic origin of infection in the human population. Thus, the virus can cause an infection in humans from either its reservoir host directly or via the various intermediate hosts [4]. All the HCoVs are well adapted in bats [9] and man gets infected from bats, mice or any domesticated animals [10].

The initial outbreak of SARS-CoV-2, epidemiologically linked to the wet Hua Nan seafood and animal market in China initially, suspected a zoonotic origin. However the first subject to develop an infection, as well as 14 out of the total 41, first confirmed cases of SARS-CoV-2 infection had no direct contact with the wet market suggesting the actual source of origin of COVID-19 at some other place and not the market [11]. The virus can, most probably, be considered as an import from some other place of origin to the market [12]. The market might have only acted as a breeding hub for viral multiplication and transmission [13].

SARS-CoV-2 shares a genetic similarity of 96% with RaTG13 which was isolated from excreta of China's horseshoe bats (*Rhinolophus affinis*) making RaTG13 the closest known relative of SARS-CoV-2 with the supporting evidence linking SARS-CoV-2 to its reservoir host, the bats [14]. However, the source of RaTG13 supposedly isolated from 'fecal swab' in 2013 is still not clear.

The Spikes of SARS-CoV-2 are longer than SARS-CoV and MERS-CoV [15] sharing a similarity of 97% and 33% respectively. The receptor-binding domain of SARS-CoV-2 displayed a genomic similarity of 74% to both RaTG13 and SARS-CoV-1 while only 19% similarity to MERS-CoV [16]. Unlike SARS-CoV-2 and SARS-CoV-1, the MERS-CoV uses dipeptidyl peptidase 4 as target receptors for host entry [17]. SARS-CoV-2 shares more sequence identity to other bats SARS-like virus such as SL-CoV-ZC45 and SL-CoV-ZXC21 (of about 88%) when compared to clinically relevant SARS-CoV and MERS-CoV (of about 80% and 50% resemblance) [18]. In fact, SARS CoV-2 showed 75.9%, 98.6%, 93.2% to 93.4%, and 91.1% sequence resemblance in the *S*, *E*, *M* and *N* genes, respectively with bat SL-CoV ZC45. However, the highly conserved sequence of RNA-dependent RNA polymerase showed only 86% resemblance to bat SL-CoV ZC45 [19] raising serious doubts about the bat reservoir origin.

Angiotensin-converting enzyme -2

Cells lining the blood vessels and alveoli express Angiotensin-converting enzyme-2 (ACE-2) receptors on their surface. Capillary-rich organs such as lungs, kidneys, intestine, stomach and brain are abundant in ACE-2 receptors [20] which are used by spikes of SARS-CoV-2 for host cell entry. The receptor-binding domain (RBD) of the S1 subunit in the viral spike interacts with the ACE-2. However, the differences in certain amino acid sequences in the RBD domain in the S1 subunit of SARS-CoV-2, not seen in SARS-CoV enhances the affinity to ACE-2 receptors, increasing its infectivity and transmission in human cells when compared to other SARS-CoV. The SARS-CoV-2 spikes, despite their broad potential to latch on ACE-receptors of different animals, have more affinity to ACE-2 receptors on primate cells including humans and the VERO monkey cells used in the laboratory, compared to other animals [21].

All species of natural bats had a variation of about 78-84% within their ACE-2 receptors while bats' ACE-2 receptors showed 80-82% difference when compared with humans and civets. Surprisingly the ACE-2 receptors of horseshoe bats were unable to act as functional receptors for SARS-CoV-1 [22]. Out of the 46 ACE-2 receptor orthologs presented from diverse species of bats, the SARS-CoV and SARS-CoV-2 failed to infect about 24 and 21 respectively, while 16 ACE-2 receptor orthologs failed to support infection with both SARS-CoV and SARS-CoV-2. Bats, therefore, exhibit the least probability of acting as a reservoir or natural host of SARS-CoV-2 as there exists a dramatic variation in the susceptibility to SARS-CoV-2 infection within bat species [23].

The ACE-2 sequence of human receptors showed more similarity to pangolins than to bats. Interestingly the spikes of SARS-CoV-2 predicted much higher affinity to human ACE-2 receptors than compared to bats or pangolins. There is no strong evidence linking the transmission of SARS-CoV-2 from pangolins to humans as the presence of CoV infection is extremely rare in wild pangolins [24]. Both the reservoir and intermediate hosts have to be clearly identified to substantiate the natural zoonotic origin of SARS-CoV-2.

The prospect of an accidental lab leak is highly probable as genetic manipulation and creation of CoV chimeras to investigate the virulence, tropism and propagation between cells of different species including humans have been conducted in the Wuhan Institute of Virology. For example, in one of the similar studies at Wuhan involving CoV, the spike of SHC014-CoV residing in Chinese horseshoe bat

populations was expressed in a wild type mouse-adapted SARS-CoV to create a chimeric SARS-CoV that had the capability to infect human airways cells via ACE-2 receptors causing considerable pathogenesis in lungs similar to SARS infection [25]. Manipulation of amino acids at a specific position (442, 472, 479, 480 and 487) in the RBD of the S1 subunit is proven to enhance the virus to bind into claw-like human ACE-2 structure with super affinity, mediating cellular entry with super efficiency into the human cells [26].

Furin cleavage site

The most fascinating feature about SARS-CoV-2 is the tactical location of the Furin Cleavage Site (FCS) with a unique polybasic site two arginine and proline amino acids right in the middle of the S1-S2 interface of the spike protein, playing a key role in the pathogenesis [27]. The protein furin, abundantly present both inside and outside human cells, precisely slices the interface of S1-S2 and expedites rapid internalization, multiplication and assembly of virus inside human cells. Newly replicated virions swiftly spread from cell to cell in the system. Unfortunately, high levels of furin in patients with underlying chronic inflammations, diabetes, cardiovascular diseases and other co-morbidities explain the increased mortality rate and complications when infected with SARS-CoV-2 [28].

The FCS in SARS-CoV-2 has several interesting and rare features absent in other CoVs [29]. The strategic presence of arginine before the FCS enables SARS-CoV-2 alternate entry mechanism into host cells via the neuropilin receptors which are more abundant than ACE-2 receptors on host cells [30]. Secondly, the FCS of SARS-CoV-2 is exposed for easy cleavage action by host proteases due to the confirmation restrain induced by the presence of the rare proline placement before the FCS sequence. The presence of hydrophobic proline before FCS separates and reveals the cleavage site from other components of the spike. Furthermore, the S1/S2 cleavage site has a longer loop with at least 4 more amino acids near the FCS enabling downstream activation and cleavage between S1/S2 by various trans-membrane serine protease facilitating easy access of SARS-CoV-2 into host cell. Some regions in FCS shares similarity with neurotoxins from Ophiophagus and Bungarus genera and Rabies lyssavirus strains [31]. Some viruses like the Marburg virus, HIV (human immunodeficiency virus), the virus causing anthrax, Ebola, influenza, etc. have FCS, responsible for viral infectivity and transmission which is absent in any of the lineages Beta CoVs. The whole data sequence of 386 CoV [32] and another database of 2956 of different virus strains did not have any FCS confirming a low probability that the SARS-CoV-2 emerged from the wild bats [33].

Specific regions on spike proteins like the FCS which is cleaved by furin or furin-like protease have significant roles in the attachment, fusion and pathogenesis in many viral diseases. In cytomegalovirus, furin mediated the host cell-viral fusion and if FCS happened to occur at inaccessible regions due to protein folding, the virus failed to enter into the host cells and was found hanging only on the cell membranes *in vitro* [34]. In paramyxoviruses, the fusion of cell membrane between virus and host to form syncytia is mediated by furin or furin-like proteinase which cleaves two FCS [35].

The chances of having an artificial insertion and manipulation resulting in SARS-CoV-2 origin are more probable and easier to explain compared to the natural mutation involving the entire amino acids sequence coding for FCS without the help of any intermediate [31]. FCS is vital in determining host range, tropism, virulence and pathogenesis as proven by the FCS insertion and manipulation experiments performed in the laboratory. For instance, induction of two FCS increased the potential of cell fusion and infectivity even in the absence of hemagglutinin-neuraminidase protein in the Sendai virus, which usually binds to sialic acid receptors of host cells to get access into the host cell [36]. Insertion of specific amino acids in spike proteins of murine hepatitis virus (MHV-A59), that only infects mice and murine cell lines, enables the virus to infect other non-murine cells like hamster, feline and monkey cells [37]. The spike proteins in CoV also have a role in promoting inflammatory response and severe acute respiratory syndrome by inducing IL-8 release from lung epithelial cells and fibroblasts [38].

Mutant SARS-CoV-2 which lacked the FCS in the spike protein showed reduced replication in human respiratory cell lines Calu3 whereas faster in Monkey cell lines Vero E6 confirming the crucial role of FCS insertion in SARS-CoV-2 in human replication and pathogenesis [39].

Evolution and natural origin

Genetic changes through different acquired mutations, which is an extremely slow process form the basis of evolution. A wild virus before infecting humans spend time jumping back and forth between its intermediate hosts accumulating mutations that finally helps to infect the human. The virus rarely infects the human in the first jump from animal hosts, as proven in the cases of MERS-CoV and SARS-CoV-1. Some time is spent jumping between the animals and humans preparing itself, indulging in 'abortive host jumping' till the virus have all the necessary mutation to attach, enter, successfully multiply and spread in human cells to cause an infection and mount an immune response [40]. The human blood specimens would contain antibodies against these viruses even before the epidemics. For both MERS and SARS-CoV-1, this was evident as proved by the presence of such antibodies (or seroconversion) in around 0.6% of the population in archived human blood from the endemic region. But in the case of SARS-CoV-2, there was no seroconversion detected in any of the samples collected and other comprehensive data regarding this is lacking [40].

Even though SARS-CoV-2 relates to RaTG13, discordant phylogenetic relationships exist between the two. The SARS-CoV-2 is not mosaic and almost half of its genome is from a new lineage with no close genetic relationship to other CoVs. Half of the spike proteins consist of multifunctional amino acids sequence needed for the successful entry of SARS-CoV-2 into human cells rejecting a recent recombinant event responsible for its emergence and RaTG13 is unlikely to be the exact variant responsible for the human outbreak [41].

The SARS-CoV-2 also lacks the presence of any posterior diversity as shown by MERS or SARS-1. For instance, the genome analysis of MERS-CoV sampled from 21 affected individuals during the previous epidemic showed three different MERS-CoV genotypes and 93% of the human cases contacted the MERS infection directly from the intermediate host, camel [42]. Similarly, in the case of SARS-1 also, phylogenetic analysis proved that extensive mutation occurred in SARS-CoV-1 while residing in the intermediate host civet [43]. However, SARS-CoV-2 lacked the posterior diversity typical of all zoonotic epidemics as all the initial patients can be surprisingly linked back to the 1st clinical case of Wuhan and phylogenetic analysis showed no extensive mutational changes during the initial outbreaks of SARS-CoV-2 [40].

Conclusion

There are many lacunae in the natural origin of SARS-CoV-2 and the possibility of SARS-CoV-2 escape from laboratory cannot be blindly ignored. The likelihood of an accidental lab leak needs to be extensively probed and assessed by an unbiased international agency and the actual starting point of the SARS-CoV-2 needs to be unearthed. The database containing details of all the CoVs maintained in the laboratory involved with infectious disease needs to be accessible to the public. Precautionary measures, compliance and maintenance of biosafety, safety audits and relocation of virology laboratories to remote places away from the public have to be strictly implemented to prevent future laboratory escapes.

Declarations

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