








# The unresolved question on COVID-19 virus origin: The three cards game?

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## Abstract

The ongoing discussion about the real origin of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) feeds acrimonious debates. Where did SARS-CoV-2 come from? Was SARS-CoV-2 transmitted in the wild from an animal to a person before exploding in Wuhan or was it an engineered virus that escaped from research or a laboratory in Wuhan? Right now, we still don't know enough whether SARS-CoV-2 is human-made or not, and lab-leak theories remain essentially speculative. Many recent studies have pointed out several plausible scenarios. Anyhow, currently, even if suspicions by some about the possibility of lab-leak hypothesis still remain, the consensus view is that the pandemic probably started from a natural source and, to determine the real origin of the SARS-CoV-2 virus, further research is needed.

## KEYWORDS

coronavirus, epidemiology, pandemics, SARS coronavirus, virus classification

Some argue the origin of Three-Card Monte, also known as the Three-card trick or Find the lady, has been around since at least the mid-nineteenth century when it was played in Paris. In French-speaking countries, the game has known as Bonneteau, in Italy Gioco delle tre Carte, in German-speaking das Kümmelblättchen, in Turkish, Bul Karayı Al Parayı "Find the Black, Get the Money." In the Three-Card Monte, the dealer mixes three playing cards, two aces, and a queen, face down on a table, and the player bets where the queen is.

If the player does not select the right card, he loses and must pay the money. However, this card game is a scam because the dealer uses all sorts of tricks, and the player never wins the game. On the true origin of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) we are probably playing the Three-Card Monte same game, everybody bets but nobody wins.

As stated by World Health Organization (WHO) COVID-19 dashboard there were 252 million confirmed cases of COVID-19 and

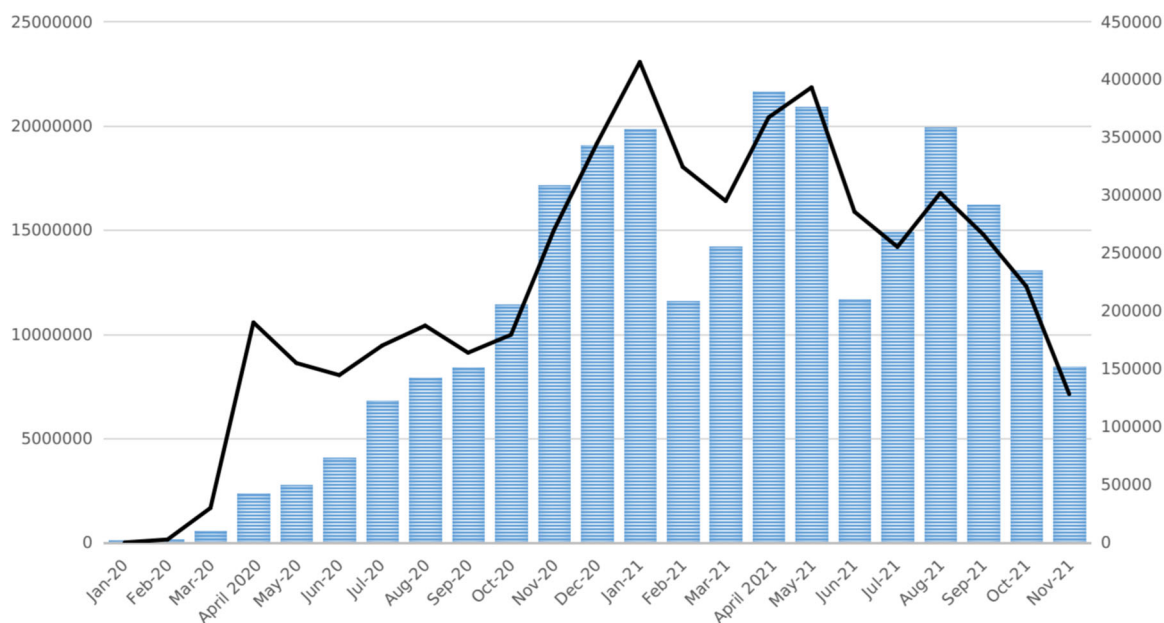
over 5 million deaths worldwide as of November 14, 2021 (see Figure 1 for details on the global monthly confirmed cases and deaths), with over 3.3 million new cases reported.<sup>1</sup> Due to the continuing evolution of SARS-CoV-2 and the generation of new variants, infections are likely to remain a problem for the time being in most countries. Moreover, the unprecedented economic contraction in 2020 around the world due to the COVID-19 pandemic, combined with the enormous loss of life, has attracted global attention on the origins of SARS-CoV-2. The ongoing discussion about the real origin of SARS-CoV-2 feeds acrimonious debates. Where did SARS-CoV-2 come from? Was SARS-CoV-2 transmitted in the wild from an animal to a person before exploding in Wuhan or was it an engineered virus that escaped from research or a laboratory in Wuhan? Fallacious origin stories about the mysterious SARS-CoV-2 origin appear in the popular press worldwide as misinformed political propaganda used by policymakers as a form of political rhetoric, such as the use of the virus as a biological weapon, with deeply harmful geopolitical implications.

Right now, we still do not know enough whether SARS-CoV-2 is human-made or not, and lab-leak theories remain essentially speculative unless someone admits that the virus was manipulated before escaping the lab accidentally.

Leaving speculative hypotheses lacking evidential support aside from real science, the WHO agreed to sponsor an independent international expert team tasked with understanding the origins of SARS-CoV-2. The WHO-China mission to Wuhan concluded a direct introduction or indirect zoonotic introduction of the virus through an intermediate host was the most plausible, however, a lab leak has not

been ruled out, and many included scores of recommendations for further study.<sup>2</sup> Last October, the WHO launched yet another investigation. WHO announced the Scientific Advisory Group for the Origins of Novel Pathogens (SAGO), a new group of experts “to define and guide studies into the origins of emerging and re-emerging pathogens of epidemic and pandemic potential, including SARS-CoV-2”, hence to make sure a disease does not cause such global disaster again.

Although there is not yet any substantial evidence for a lab leak, and most scientists support a natural origin of the virus, by a jump to humans from bats, if it was a direct spillover—or, more likely, through an intermediate mammal, researchers have looked into genetic features of SARS-CoV-2 bioengineering signals. A team of scientists combed through the genome sequence for any signs of lab tinkering and determined that were not engineered genetic elements and they concluded that SARS-CoV-2 was not a laboratory construct<sup>3</sup> SARS-CoV-2 contains a key mutation in the spike protein: the “furin cleavage site”, caused by an out-of-frame insertion. Furin cleavage site, consisting of four amino acids RRAR which are encoded by 12 inserted nucleotides, is necessary for activation of the spike glycoprotein responsible for virus entry and cell fusion enhancing infectivity and virulence. Some lab-leak proponents have suggested, that this site has been introduced via genetic engineering into the virus as similar features have not been found in any other SARS-like coronavirus. In fact, a characteristic feature of this site is an arginine doublet encoded by the CGGCGG codons, that is not found in any of the canonical furin sites (R-X-R/K-R) in other viral proteins expressed by a wide range of viruses.<sup>4</sup> As many scientists have since pointed



**FIGURE 1** Global COVID-19 cases and deaths monthly reported, as of 18 November 2021. Series 1 on the left indicates the number of confirmed cases (stacked columns) and series 2 on the right indicates the number of confirmed deaths (black line). Monthly values have been obtained by daily reports on a 7-day rolling average. Raw data on confirmed cases and deaths for all countries are sourced from the COVID-19 Data Repository by the Center for Systems Science and Engineering (CSSE) at Johns Hopkins University, available at <https://github.com/CSSEGISandData/COVID-19>

out, a cleavage site that it's not cleaved very efficiently by furin it's incredibly unlikely that was engineered. Gallaher showed that in the spike gene of both SARS-CoV-2 and bat coronavirus HKU9-1 there is a near-identical nucleotide sequence and upstream of this sequence are present short oligonucleotide breakpoint sequences, that facilitate recombinational events.<sup>5</sup> This mechanism provides a natural explanation for an out-of-frame insertion of a furin cleavage site in SARS-CoV-2 fixed by natural selection. Further complicating the lab-leak scenarios are that serially propagating SARS-CoV-2 in Vero E6 cells leads to deletions in the furin cleavage site.<sup>6</sup> On the other hand, RmYN02 from bats living in Southern China contains the insertion of multiple amino acids at the S1 and S2 junction site of the subunits of the spike, providing strong evidence that such insertion events can occur naturally in betacoronaviruses.<sup>7</sup>

Most emerging infectious diseases in humans begin with a spillover caused by interspecies barrier breakthrough of animal-origin pathogens, as was seen with influenza epidemics, HIV, Ebola, SARS in 2002, and the Middle East respiratory syndrome (MERS) outbreak in 2012. The zoonotic event is the most likely scenario of the natural origin of SARS-CoV-2. Defining the precise natural origin of the virus would help to decline alternative hypotheses (see Worobey<sup>8</sup>). Multiple bat viruses are closely related to SARS-CoV-2 including RaTG13 obtained from *Rhinolophus affinis* in the southern Chinese province of Yunnan. RaTG13, despite sharing 96.2% sequence identity with SARS-CoV-2, its affinity for ACE2 is very limited, as the spike receptor-binding domain (RBD) shows low sequence similarity with that of SARS-CoV-2 thus suggesting that this virus is not the proximal ancestor. Like SARS and MERS, the possible involvement of intermediate host as a plausible conduit for transmission to humans has been considered. Guangdong Pangolin-CoV genome is very closely related to that of SARS-CoV-2 sharing 92.4% sequence similarity, thus pangolin could be responsible for the zoonotic event. Several species of mammals (including ferrets, cats, mink, etc.) are capable of being infected by SARS-CoV-2, thus it is possible that SARS-CoV-2-related viruses cross the species barrier between humans and animals.<sup>9</sup> However, WHO reported that 80 000 wildlife and farm animal samples from China had been tested, resulting negative for SARS-CoV-2, and most importantly, all SARS-CoV-2 like viruses isolated from bats in China and Japan Thailand, do not have a spike protein that can bind ACE2 and allow entry into human cells. Last September, a very considered review of all virological and epidemiological evidence regarding the origins of the SARS-COV-2 has been published by a group of experts in virus evolution and molecular virology. Although the authors could not be entirely ruled out a "laboratory escape" scenario, they make a strong case for the animal-to-human cross-species spillover followed by ongoing adaptation in humans.<sup>10</sup>

Recently, a team of researchers led by Marc Eloit of the Pasteur Institute, discovered that bats from three *Rhinolophus* species living in caves in Northern Laos close to the Southwest China border, host three Sarbecovirus genomes, called BANAL-52, -103, and -236, the closest relatives to SARS-CoV-2 found to date. They obtained this result by sampling blood, saliva, feces, and urine from 645 bats of 46 different species.<sup>11</sup> In the preprint under review on Research Square

on September 17, they showed that the RBD of these new viruses has an affinity for human ACE2 receptors closer to SARS-CoV-2 than that of any other known bat virus. In addition, using crystallography, the scientists showed BANAL-236 structure identical to the RBD of SARS-CoV-2 and the ability of BANAL-236 spike protein to infect human cells producing ACE2. Infectious BANAL-236 was also cultured in Vero cells, providing a rare virus isolate for a bat SARS-CoV-2 like virus to be used to study how pathogenic the virus is in animal models. Phylogenetic analysis suggested that the SARS-CoV-2 genome could be a patchwork of different genomes, including those of BANAL viruses, and the already known RmYN02, RpYN06 and RaTG13. This implicates that SARS-CoV-2 likely originated from recombination of viruses circulating in different species of *Rhinolophus* bats, which do not migrate far but might infect other species that share roosts and jump around South China and Southeast Asia. This discovery offers insight into the origins of the pandemic and it provides more evidence of similar viruses circulating in bats naturally, bolstering the "natural zoonotic origin" of SARS-CoV-2. Despite this, researchers still raise unresolved questions of how a progenitor of the virus could have traveled to Wuhan and of the absence of the furin cleavage site in the BANAL-236 spike. It is possible that the pandemic virus' progenitor could have picked up its site after spillover into humans or another intermediate host, or alternatively, that accumulation of a lot of mutations could have generated it. It is also possible that such sites might be found in bat viruses, and simply they have not yet been traced because of insufficient sampling, thus intrinsically posing a future risk of possible direct transmission to humans.

Another preprint, that has not completed peer review, by Wu and colleagues, found no SARS-CoV-2-related viruses in 13 064 bats between 2016 and 2021 at 703 locations across China, suggesting that viruses considered closest to SARS-CoV-2 are "extremely rare" in bats in China.<sup>12</sup> Authors concluded that further research should be done to determine whether the SARS-CoV-2 virus originated in China.

In conclusion, even if suspicions by some about the possibility of lab-leak hypothesis still remain, the consensus view is that the pandemic probably started from a natural source. And the story goes on.

## CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

## AUTHOR CONTRIBUTIONS

*Conceptualization:* Alessandra Borsetti, Fabio Scarpa and Massimo Ciccozzi. *Writing—original draft preparation:* Alessandra Borsetti and Massimo Ciccozzi. *Writing—review and editing:* Alessandra Borsetti, Fabio Scarpa, Antonello Maruotti, Fabio Divino, Giancarlo Ceccarelli, Marta Giovanetti and Massimo Ciccozzi. *Supervision:* Massimo Ciccozzi.

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