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Might SARS-CoV-2 Have Arisen via Serial Passage through an Animal Host or Cell Culture?

A potential explanation for much of the novel coronavirus' distinctive genome

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Abstract

Despite claims from prominent scientists that SARS-CoV-2 indubitably emerged naturally, the etiology of this novel coronavirus remains a pressing and open question: Without knowing the true nature of a disease, it is impossible for clinicians to appropriately shape their care, for policy-makers to correctly gauge the nature and extent of the threat, and for the public to appropriately modify their behavior. Unless the intermediate host necessary for completing a natural zoonotic jump is identified, the dual-use gain-of-function research practice of viral serial passage should be considered a viable route by which the novel coronavirus arose. The practice of serial passage mimics a natural zoonotic jump, and offers explanations for SARS-CoV-2's distinctive spike-protein region and its unexpectedly high affinity for angiotensin converting enzyme (ACE2), as well as the notable polybasic furin cleavage site within it. Additional molecular clues raise further questions, all of which warrant full investigation into the novel coronavirus's origins and a re-examination of the risks and rewards of dual-use gain-of-function research.

1 Introduction

To date, the origins of SARS-CoV-2 remain in doubt, and its behavior enigmatic: It has been reported that "the virus acts like no microbe humanity has ever seen."[1] Although based on sequence analysis many prominent virologists and other eminent scientists have concluded that the novel coronavirus causing the current pandemic was not designed or manipulated in a laboratory and was the result of a natural zoonotic jump,[2] this assertion fails to fully account for all possible origins of two unique genomic characteristics found in SARS-CoV-2, and ignores the long history of serial passage as a method to manipulate viral genomes. The long-standing practice of serial passage is a form of gain-of-function research that forces zoonosis between species, and requires the same molecular adaptations necessary for a natural zoonotic jump to occur within a laboratory, leaving the same genetic signatures behind as a natural jump but occurring in a much shorter period of time.

The genetic signatures in question includes two distinctive features possessed by SARS-CoV-2's spike-protein: the unique sequence in the receptor binding domain (RBD), a region known to be critical for SARS-CoV-2's utilization of human angiotensin converting enzyme (ACE2), which is the cell surface receptor used by both SARS-CoV and SARS-CoV-2 for fusion with target cells and subsequent cell entry. The second feature is the presence of a polybasic furin cleavage site, which is also known as a multibasic cleavage site (MBS)—a four amino acid insertion with limited sequence flexibility—within the coronavirus's novel spike-protein, that is not found in SARS-CoV or other lineage B coronaviruses. This furin cleavage site, which is poly or multibasic by definition since its composed of multiple basic amino acids, is an important virulence feature observed to have been acquired by fusion proteins of avian influenza viruses and Newcastle Disease Virus either grown under experimental conditions or isolated from commercial animal farms—settings that mimic the conditions of serial laboratory passage. In fact, no influenza virus with a furin cleavage site has ever been found in nature,[3] and it is a feature that has been thoroughly investigated in the literature since it appears to allow the influenza viruses that carry it to establish a systemic multiorgan infection using different cell types including nerve cells,[3] is correlated with high pathogenicity, and also plays a key role in overcoming the species barrier.[4] More generally, despite the fact that not all serially passed viruses have demonstrated an increase in pathogenicity, the fact remains that every highly pathogenic avian influenza virus, defined by having a furin cleavage site, has either been found on commercial poultry farms that create the pseudo-natural conditions necessary for serial passage, or created in laboratories with gain-of-function serial passage experiments.[3]

Although they only emerge under artificial conditions in influenza viruses, these furin cleavage sites are found within several branches of the coronavirus family tree. However SARS-CoV-2 is the only lineage B coronavirus found with one, and the only other coronaviruses known to have them are only at most 60% identical to this novel coronavirus.[5] An intriguing clinical correlate is that furin cleavage sites within influenza viruses are associated with lymphopenia in infected mice, and with neurological conditions following replication in the brains of ferrets,[6] both of which are clinical manifestations observed in hospitalized patients infected by SARS-CoV-2 and suffering from COVID-19.[1] This indicates that furin cleavage sites may be an example of the convergent evolution that dominates virus–host interactions, since viral proteins evolve convergently and often accumulate many of the same linear motifs that mediate many functionally diverse biophysical interactions in order to manipulate complex host processes.[7] It is possible that this novel coronavirus gained its furin cleavage site through recombination in an intermediate host species, however there are also two laboratory processes that may have imbued SARS-CoV-2 with its furin cleavage site which will be discussed below.

Without incorporating the historical and biological implications of serial viral passage either through lab animals in vivo or through cell cultures in vitro, it is impossible to comprehensively evaluate whether SARS-CoV-2 is the result of a laboratory leak or a natural zoonotic jump. Moreover, despite the published consensus being that SARS-CoV-2 arose naturally, because these publications universally ignore the scenario of the widely used practice of laboratory serial passage, this latter scenario deserves a thorough investigation. Especially since serial passage through a live animal host simply forces the same molecular processes that occur in nature to happen during a zoonotic jump, and in vitro passage through cell culture mimics many elements of this process—and neither necessarily leaves any distinguishing genetic traces.

2 The History of Viral Serial Passage

The dual-use gain-of-function research tool of serial passage was first applied to a strain of H1N1 Swine Flu, a variant of the pandemic influenza virus that was genetically modified before it either leaked out of a Soviet lab or was introduced as part of an attenuated vaccine trial in 1977. Although no one has ever taken responsibility for the introduction of this virus, it would become the first known example of a virus created by serial passage leaving a lab, which was later determined due to its inexplicable genetic distance from any known sister strain.[8] This extra distance would be expected since serial passages artificially accelerates genetic divergence between taxa, resulting in the accumulation of genetic distance at a much faster rate than it occurs in a natural setting.

Then in 1979, just 2 years after the introduction of this modified H1N1 Swine Flu, a different Soviet lab leaked weaponized anthrax out through an improperly maintained exhaust filter, and Soviet authorities convincingly blamed the deaths on contaminated local meat. This cover up withstood a formal inquiry conducted in 1986, and was not revealed to be a fabrication until 1992, when an analysis of dispersion patterns revealed that the victims were not those working with the supposedly contaminated meat, but instead all lived downwind from the Sverdlovsk weapons lab and its improperly maintained exhaust vent. Therefore, there is a history of denying laboratory leaks on the commercial meat industry that dates back about 40 years, an effective excuse that provided the Soviets with an alibi that held up for nearly 2 decades.

The Soviet strain of serially passaged H1N1 Swine Flu was likely being developed as part of a vaccine program, one of the humane goals of gain-of-function research that exist alongside riskier and more troublesome ones like developing bioweapons. Its emergence ignited the debate between the risks and rewards of dual-use gain-of-function research—causing it to became the poster virus for the dangers this protocol posed.[8]

This debate would largely fade in the decades that followed, until two separate teams used genetic manipulation followed by serial passage between ferrets to create mammal-transmissible H5N1 Bird Flu strains of influenza virus in 2011 that had the gain-of-function of being transmissible by aerosol. The first team was led by Dr. Ron Fouchier and conducted at the Erasmus Medical Center in the Netherlands, and demonstrated that as few as five mutations prior to serial passage were sufficient to create a modified strain of the H5N1 Bird Flu that could be transmitted by aerosol while remaining highly lethal.[9] The creation of this highly virulent strain that was said by a reporter to be able to "make the deadly 1918 pandemic look like a pesky cold,"[10] and was contentious enough to cause the scientists working on them to prepare for a media storm[11]—a storm that rolled in on the back of a second similar experiment.

Instead of only tweaking the H5N1 Bird Flu in a few places before serial passage, Dr. Yoshihiro Kawaoka of the Universities of Tokyo and Wisconsin used genetic engineering to combine genes from the H1N1 Swine Flu as well as the H5N1 Bird Flu to create a chimeric virus that was then serially passed through ferrets, creating another airborne virus with potentially pandemic properties.[12] Both experiments created a modified genome that appeared to be the result of natural, albeit accelerated, selection since the process of serial passage forces the mutations selected for in natural zoonotic jumps, and masks the direct genetic engineering done on the viruses. These experiments were viewed by many as being sufficiently dangerous that they should not be published,[13] however they were both eventually released with certain methodological and sequence details left out.

In the years that followed, gain-of-function serial passage through ferrets was used to increase the virulence of the H7N1 Bird Flu as well as allowing for its aerosol transmission without first introducing any mutations.[14] Additionally, the H1N1 Bird Flu was also found to become airborne and increase in virulence after in vivo passage through swine.[15, 16] And although serial passage in the laboratory does not invariably increase viral pathogenicity, highly pathogenic influenza viruses all contain furin cleavage sites,[16] which only emerge after serial passage in laboratories or pseudo-naturally on commercial animal farms.

The process of sequential passage through animal hosts or cell cultures leaves a genome that appears natural and not purposefully manipulated since it effectively mimics the natural process of zoonosis, and leaves a genome that appears to be the result of natural selection so long as its relationship to related strains of virus is ignored. However, the artificial generations added by forced serial passage creates the artificial appearance of evolutionary distance, which was the characteristic of the H1N1 Swine Flu Soviet leak in the 1970s that lead researchers to conclude it had been constructed in a lab, and is exactly what is found with SARS-CoV-2, which is distant enough from any other virus that it has been placed in its own clade.[17]

2.1 Serial Passage and Its Molecular Signatures

Although serial passage mimics many of the natural zoonotic processes that occur during a natural zoonotic jump, because serial passage artificially condenses a natural phenomenon into a small temporal window, some subtle differences can be found. In addition to the inexplicable genetic distance from its sister strains, which screams out for an intermediate relative to complete the phylogenetic picture, SARS-CoV-2 has a remarkably strong affinity for spike-protein binding to ACE2—some 10–20 times higher than SARS-CoV's.[18] That affinity may have emerged after mutational events either in an intermediate natural host or after a zoonotic jump into humans that theoretically could have occurred earlier than the first documented infection, which would give it time to increase that significantly. So logically, it could also have emerged via selection after serial passage through laboratory cell cultures or laboratory animals as well. And regarding the second distinctive feature found in the novel coronavirus: If other viruses have been observed to acquire furin cleavage sites by passage under experimental laboratory conditions, then such a mechanism is theoretically possible for SARS-CoV-2 as well.[2]

In the case of influenza viruses like those mentioned above, their gain-of-function furin cleavage sites are thought to be a result of two different molecular processes. The first is either nucleotide insertions or substitutions that are able to be rescued and then eventually selected for due to the high multiplicity of infection found in serial passage protocols.[19] And the second is the recombination of multiple viral RNAs inside a host cell,[20] which may also include additional viruses introduced through accidental laboratory co-infections.

Unlike influenza viruses, serial passage through ferrets has not been recorded in the literature for coronaviruses. However, since several branches of coronavirus have furin cleavage sites, a molecular pathway for their emergence must exist and may reemerge during serial passage. Several factors weigh into the probability that coronaviruses can gain furin cleavage sites following serial passage: The frequency of evolutionary motifs meant to deal with virus–host interactions that are often shared between viruses, the observations that when the infectious bronchitis coronavirus (IBV) coronavirus is serially passed through chickens it developed notable mutations along its spike-protein genes,[21] and the fact that when a lineage A bovine coronavirus was subject to in vitro serial passage through cell lines, a 12-nucleotide insert found within only a small minority of the pooled viruses spike-protein region was strongly selected for and quickly emerged as the dominate strain.[22] These findings all point to the possibility that SARS-CoV-2 may have gained its furin cleavage site the same way influenza viruses do—through the in vivo serial passage between the live hosts that presents the immune challenges and intense selective pressure necessary for the recombination and mutations that lead to its emergence to occur. And just like influenza viruses are only able to preserve their furin cleavages in artificial environments since the heightened virulence they impart kills their hosts before they can propagate in a natural setting, based on the known taxonomy lineage B coronaviruses do not appear to be able to support furin cleavages in nature.

There is no doubt that the acquisition of the furin cleavage site was one of the key adaptations that enable SARS-CoV-2 to efficiently spread in the human populations compared to other lineage B coronaviruses, and provides a gain-of-function.[23] In addition to the possibility of obtaining a furin cleavage site through natural recombination in a secondary host or through serial passage either in a laboratory or on a commercial farm, one could have been spliced directly into the novel coronavirus's backbone in a laboratory using classic recombinant DNA technology that has been available for nearly 20 years. This allows for the removal of the restriction site junctions that are the telltale sign of direct genetic manipulation and permits reassembly without introducing nucleotide changes —creating a virus without any evidence of manipulation using the aptly named "No See'm technology."[24] So although the entire spike-protein RBD was not assembled from scratch, it is certainly plausible that the 12-nucleotide-long furin cleavage site could have been spliced directly into SARS-CoV-2. Furin cleavages already have been successfully spliced into other coronaviruses, including the IBV,[25] and even into SARS-CoV, where it increased cell-to-cell fusion in in vitro experiments that only examined only the spike-protein's function, which would presumably heighten its infectivity in vivo.[26]

Moreover, when a furin cleavage site was introduced to the IBV coronavirus spike-protein via recombination, just like influenza viruses hosting this feature, it appeared to impart it with increased lethality as well as inflict neurological symptoms that had never previously been reported in studies of the murine IBV coronavirus.[25] The presence of this cleavage site also increased damage to the respiratory and urinary systems, paralleling SARS-CoV-2 systemic multiorgan symptoms—especially reports that infection with the novel coronavirus not only targets the lungs where it binds to ACE2 receptors, but also the entire cardiovascular system,[27] the nervous system,[28] and our kidneys as well.[29] It might be more than a coincidence that the Vero cells often used in serial passage are derived from kidney epithelial cells extracted from African green monkeys, which have ACE2 receptors very similar to those found in humans and would be shared by the humanized mice that are also used for serial passage research.

2.2 Natural Origin, or Gain-of-Function Lab Escape?

Gain-of-function research on bat-borne coronaviruses has been ongoing for nearly a decade everywhere from the University of North Carolina to the Wuhan's Institute of Virology, which is supported by related facilities such as Wuhan's Center for Disease Control and Prevention as well as Wuhan University. A coronavirus that targets the ACE2 receptor like SARS-CoV-2 was first isolated from a wild bat in 2013 by a team out of Wuhan. This research was funded in part by EcoHealth Alliance,[30] and set the stage for the manipulation of bat-borne coronavirus genomes that target this receptor and can become airborne. Many more viruses have been collected in Wuhan over the years, and one research expedition captured as many as 400 wild viruses,[31] which were added to a private repository that has since grown to over 1500 strains of virus,[32] meaning that the Wuhan Center for Disease Control and Prevention has a massive catalogue of largely undisclosed viruses to draw from for experiments. And in subsequent years, EcoHealth Alliance received funding for project proposals outlining gain-of-function research to be done in Wuhan, hoping to use cell cultures and humanized mice as well as "[spike]-protein sequence data, infectious clone technology, in vitro and in vivo infection experiments and analysis of receptor binding"[33] to manipulate bat coronavirus genomes—all of which are consistent with the wet-work that would be needed to engineer this novel coronavirus in a laboratory. But for whatever reason, the Wuhan Institute of Virology has refused to release the lab notebooks of its researchers, which are ubiquitous in even the simplest laboratories and are expected to be meticulously detailed given the sensitive and delicate work that takes place in BSL-4 research labs intent on documenting their intellectual property, despite the fact that these notebooks would likely be enough to exonerate the lab from having any role in the creation of SARS-CoV-2.[34]

Although it does not prove a laboratory origin, another gain-of-function experiment demonstrates one possible step along the way to engineering SARS-CoV-2: the synthetic reconstruction of the SARS coronavirus to impart this virus with a high affinity for ACE2. This involved isolating a progenitor coronavirus from civets and then serially passing it through mammalian ACE2 receptor-expressing cells—serial passage through host cell lines instead of entire hosts, which imparted a strong affinity for ACE2,[35] and another novel strain of coronavirus that was also presumably airborne. A few years after this study, more gain-of-function research was performed that involved the creation of a chimeric bat-borne coronavirus by directly manipulating the bat coronavirus spike-protein gene,[36] which created a coronavirus so virulent that it evoked the following dire warning from Simon Wain-Hobson, a virologist with the Pasteur Institute in Paris: "If the [new] virus escaped, nobody could predict the trajectory."[37]

Although SARS-CoV-2's efficient solution for ACE2 binding has been accurately described as something that could not be intentionally engineered nucleotide-by-nucleotide,[2] it could well be selected for after serial passage through ferrets or cell cultures in a lab. The only origin for the SARS-CoV-2 spike-protein RBD that the sequence data excludes is the deliberate manufacturing and introduction of the entire SARS-CoV spike-protein RBD sequence to create SARS-CoV-2. Otherwise, there are no genetic data to distinguish among natural and engineered possibilities at the present time.

2.3 Ferreting Out the Signs of Serial Passage

Curiously, studies examining SARS-CoV-2's infectivity in ferrets found that it spreads readily among them, and also appears airborne in that animal model.[38] This lends support to the idea that ferrets may have been used for serial passage since viruses typically take a significant many months if not years to acclimate enough to spread at all among any new species, nonetheless become airborne, which requires further mutations.

This relationship was further supported by reports out of the Netherlands that the novel coronavirus had spread among thirteen different mink farms there, and also to at least one farm in Denmark[39] and to another in Spain where 87% of the mink were infected.[40] Minks are a closely related subspecies of ferret that can produce fertile offspring together, and so the fact that not only did the virus spread to fifteen different farms in three countries, but also appears to have spread from minks into farm workers[41] indicates that accidental commercial serial passage through minks could have played a role in its creation, as an alternative to laboratory ferrets. Nevertheless, regardless of where any possible serial passage occurred, the fact that SARS-CoV-2 spreads from humans to minks and then back to humans demonstrates a high affinity for both species, despite neither nominally being a natural reservoir. Further support for the possibility that serial passage through lab ferrets or throughout mink farms played a role in the genesis of this novel coronavirus is provided by a preprint that notes the obvious ease with which it passes through the air between ferrets, since SARS-CoV-2 was transmitted through the air to three out of four indirect recipient ferrets monitored for airborne passage of the novel coronavirus.[42] It seems reasonable to think that SARS-Cov-2's apparent affinity for ferrets and minks should lead to an investigation of mink farms in the Hubei province were the novel coronavirus was discovered, since a viable pathway for its emergence could be infected bats defecating on commercial mink farms, which would loosely parallel the emergence of MERS-CoV from herds of camels following putative fecal contamination by local bats.[43]

The prospect that serial passage through lab animals or on commercial farms may have played a role in the creation of SARS-CoV-2 is also raised by an April 2020 preprint, which appears to have been retracted after Chinese authorities implemented the censorship of any papers relating to the origins of the novel coronavirus.[44] This paper found that coronaviruses that target the ACE2 receptor bind with ferret cells more tightly than any other species except the tree shrew, which only scored about 2% higher. Tree shrews have also been used for serial viral passage, and have been promoted as a preferable animal host for laboratory experimentation since they are cheaper, smaller, easier to handle, and closer to humans evolutionarily and physiologically than ferrets.[45] However, one does not exclude the other as a possible host, and a recent preprint examining SARS-CoV's binding affinity in humans raises additional questions about its initial emergence. It found that the novel coronavirus appears to be far more adapted to human ACE2 receptors than those found in bats, which is unexpected given that bats are the virus's assumed source, and which lead the lead research to observe that SARS-CoV-2 was perfectly adapted to infect humans since its first contact with us, and had no apparent need to for any adaptive evolution at all.[46]

Although the novel coronavirus also appears to have a high affinity for the pangolin ACE2 receptor,[47] phylogenetic analysis of the neutral sites that best determine shared heritage[48] and a distinctive amino acid sequence both indicate that pangolins are unlikely to have served as an intermediate host,[47] so this affinity is likely due to the convergent motifs that often mark viral evolution and not shared heritage. The unexpected immediate affinity for humans was also reflected by another preprint, which observed that SARS-CoV-2 appeared just as adapted to humans at the very start of its epidemic as SARS-CoV was in the latest stages of its emergence,[49] an unexpected finding since viruses are expected to mutate substantially as they acclimate to a new species.[50] SARS-CoV-2's muddled origins are made even more Gordian by a study published March 2018 that examined people who live in villages about a kilometer away from bat caves. This study revealed that only 2.7% of those villagers had antibodies indicating any past exposure to bat coronaviruses. The authors also sampled people living in Wuhan, and found no evidence of exposure to SARS-CoV-like coronaviruses at all.[51]

This means there is very little serological evidence of any exposure to these coronaviruses even in Chinese villagers living in close proximity to bat caves, and at the epicenter of the current outbreak—no previous exposure was found at all. These data do not support the idea that SARS-CoV-2 was circulating in humans prior to the outbreak began in Wuhan in the early winter or fall of 2019, making a zoonotic jump even more unlikely since natural jumps leave wide serological footprints in their new host populations as early variants of a prospective virus make limited and unsuccessful jumps into individuals of the new host species, a trial-and-error that must occur before mutations that allow adaptation to a new host species are selected.[50] However these results do not rule out a much earlier jump into humans somewhere outside Hubei province, an alternative that is awaiting empirical support.

Taken together, the available evidence does not point definitively toward a natural origin for SARS-CoV-2, rather, much of it is more consistent with what would be found if the novel coronavirus had arisen from serial passage of a “precursor” progenitor virus in a lab, or from bats infecting a commercial mink farm somewhere in China, which would also provide the conditions for serial passage. However, more evidence is required before a conclusive judgement can be made one way or the other.

Further research around SARS-CoV-2's affinity to ferrets and minks, as well as other possible intermediate hosts seems warranted, and certainly the examination of all past gain-of-function serial passage research by the scientific community at large should occur to determine what other definitive genomic signatures serial passage leaves besides the creation of furin cleavage sites, in case more of those can be found in this novel coronavirus. Two additional unique genomic signature are already being researched, as one preprint indicates that SARS-CoV-2 possesses a genomic region not found in other coronaviruses that appears to cloak the novel coronavirus from white blood cells, a characteristic also found with HIV.[52] And the second preprint identifies a region on the spike-protein gene found in no other bat-borne coronavirus that is nearly identical to superantigenic and neurotoxic motifs found in some bacteria, which may contribute to the immune overreaction that leads to the Kawasaki-like multisystem inflammatory syndrome in children, and cytokine storms in adults.[53] Given the unique traits found in SARS-CoV-2 and all the open questions there still are around its emergence, until either a natural or laboratory origin is conclusively demonstrated both avenues should be robustly investigated by the scientific community.

3 Conclusions and Outlook

The history of gain-of-function research is one of science's most significant and troubling, especially since the Nuremberg Code, research scientists' Hippocratic Oath, dictates that experiments that could endanger human life should only occur if the potential humanitarian benefits significantly outweigh the risks.[54] It seems ill-advised to rule out the possibility that gain-of-function techniques such as serial passage may have played a role in the creation of SARS-CoV-2 until more definitive data are collected, and when the Center for Arms Control and Non-Proliferation has calculated that the odds that any given potential pandemic pathogen might leak from a lab could be better than one in four.[55]

The release of the H1N1 Swine Flu in 1977 first initiated the discussion about the moral and physical hazards involved with dual-use gain-of-function research, and it was the creation of extraordinarily virulent H5N1 Bird Flu strains—using the same technique of serial passage through an animal host in a lab—that contributed to the NIH imposing a moratorium on dual-use gain-of-function research from 2014 until 2017, after which it was relaxed explicitly to allow influenza strains as well as coronaviruses to be studied. This moratorium was meant to limit "the potential to create, transfer, or use an enhanced potential pandemic pathogen."[56] However, just as an increased pace of research into influenza vaccines increased the odds that a leak would occur leading up to the 1977 release of H1N1 Swine Flu, which is the most often cited as originating from a laboratory leak,[8] it would follow that an increased pace of research into coronaviruses over the past few years would have increased the odds that a lab leak of one would occur; after all, these viruses were pinpointed back in 2006 as a viable vector for an HIV vaccine[57] and research into a pan-coronavirus vaccine has been ongoing for decades.

And whether or not gain-of-function research is determined to have played a role in SARS-CoV-2's emergence, the fact that it creates opportunities for pandemic viruses to leak out of labs calls for a re-examination of the moratorium against this practice, because the emergence of this novel coronavirus has demonstrated that the international public health community is not prepared to handle the leak of a pandemic virus. Furthermore, none of the gain-of-function research conducted since 2014 has provided humanity with any tools at all to fight back against the ongoing pandemic caused by this novel coronavirus.

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Conflict of Interest

The authors declare no conflict of interest.