SARS-CoV-2 Reveals that Chimeric Agents are the Bioweapons of the Future

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Abstract

Bioweapons programs have existed since their development during the Cold War. These biowarfare programs initially utilized naturally occurring pathogens capable of infecting crops, livestock populations, and human populations. Anthrax is a widely exploited bioagent responsible for attacks ranging from the Germans' deployment in World War I to the mailing of anthrax through the postal service in attempts on U.S. senators' lives. With the development of genetic manipulations, the Soviet Union began modifying anthrax to resist detection and treatment. With the continued advancement of science and technology, a new bioagent has entered the scene – the man-made chimeric virus. Chimeric viruses typically only infect a certain species, however with genetic alteration they develop characteristics required to infect other species. *SARS-CoV-2* and its apparent genetic changes could prove a prime example of how a bat virus underwent genetic mutations that allow it to infect humans. Ultimately, *SARS-CoV-2* reveals how chimeric viruses are the bioweapons of the future.

SARS-CoV-2 Reveals that Chimeric Agents are the Bioweapons of the Future

Poisons have long been utilized in assassinations throughout history, and the rise of microbiology in the late 1800's fostered the development of comprehensive bioweapons programs during the World Wars. After the World Wars in the Cold War Era, while the world was transfixed on the race to get the first man in space and the race for nuclear superiority, another, more covert race was taking place: the race to develop the most lethal bioagents and to subsequently stockpile bioweapons capable of mass destruction. In response to the rapid developing and stockpiling of bioweapons, nations – including the United States – came together to form the Biological Weapons Convention (BWC), which sought to eliminate research of bioagents intended for use in weapons of mass destructions, demanded the halt of stockpiling bioweapons, and necessitated the proper disposal of previously constructed bioweapons. However, while the major powerhouse nations of the bioweapons race signed and ratified the Biological Weapons Treaty, it would benefit the United States defense organizations to assume that Russia and China have not halted the research and production of lethal bioagents. Thus, to ensure the effective upholding of U.S. biodefense and biosecurity, the question remains as to the focus of modern bioweapons research.

While the origin of the *SARS-CoV-2* virus is still widely disputed, the specific genetic alterations in this virus grant valuable insight as to the potential for the future of bioweapons research. Originally, the *SARS-CoV-2* virus backbone seems to have been derived from the bat RaTG13 virus, which raises the question as to how this bat virus is capable of infecting humans and leading to a global pandemic. The answer lies in several genetic mutations in the *SARS-CoV-2* virus that gave rise to a furin cleavage site, allowing the *SARS-CoV-2* virus the ability to infect human lung cells and lead to a systemic infection in humans. Interestingly, furin cleavage sites

are common in the most infectious bioagents – such as the widely weaponized pathogen anthrax – as the cleavage site allows the bioagents to receive activation signals from the furin protease enzyme, a molecule that is ubiquitous in all human tissues (Braun, 2019). While *SARS-CoV-2* may not have been intentionally developed as a bioweapon, its expressed genetic modifications demonstrate that laboratories are researching and developing chimeric viruses and the biodefense implications of this research must be addressed.

It is important to note that the purpose of this paper is not to claim that China released *SARS-CoV-2* as an act of biowarfare or even to develop it as a biowarfare agent. Rather, it is the intent of this paper to analyze the potential for the genetic modifications exhibited by the *SARS-CoV-2* virus to translate to biowarfare and the implications of that translation. The Covid-19 pandemic has wreaked havoc on nations around the globe, and the scientific community has devoted countless hours and resources to investigate the genetic mutations that granted this virus human ACE2 receptor binding specificity. Thus, it is only logical that nations developing modern bioweapons programs would also be researching this and looking for the potential to translate these alterations into biowarfare agents. This paper seeks to establish why chimeric viruses are one of the focuses of modern-day bioweapons programs.

Introduction to the Biology and Classification of Bioagents

CDC Categories of Bioterrorism Agents

The Center for Disease Control and Prevention (CDC) distinguishes three different categories of biological agents: Category 1, Category 2, and Category 3 bioterrorism agents. Category 1 bioterrorism agents are the highest threat to national security and have the highest priority for study. Agents within this category are defined as those pathogens which “can be easily disseminated or transmitted from person to person; result in high mortality rates and have

the potential for major public health impact; might cause public panic and social disruption; and require special action for public health preparedness" (Center for Disease Control and Prevention, 2018). These agents include pathogens such as anthrax, botulinum, smallpox, and plague. Category 2 biowarfare agents have the second highest priority and are defined as those agents that "are moderately easy to disseminate; result in moderate morbidity rates and low mortality rates; and require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance" (Center for Disease Control and Prevention, 2018). Finally, Category 3 bioagents are defined as those "emerging pathogens that could be engineered for mass dissemination in the future because of availability; ease of production and dissemination; and potential for high morbidity and mortality rates and major health impact" (Center for Disease Control and Prevention, 2018).

Pathogens' Lethality Increases Due to Modern Genetics

The rise of modern genetics has not only increased the potency of Category 1 agents, but it has also created the potential for those pathogens originally in Category 2 and 3 to be elevated to Category 1 status as the genetic modifications result in increased lethality. The Soviet Union's Biopreparat program is a prime example of how modern genetics was applied to produce increasingly deadly bioagents. The Biopreparart had three goals when genetically altering a pathogen (Davis, 1999).

First, the pathogen should be altered in a manner that would permit it to withstand harsh environments. This would allow a pathogen to be deployed virtually anywhere in the world by enhancing its resiliency. Pathogens should also be able to avoid detection by surveillance systems. One such modification could occur through the alteration of primer sequences within the genetic code of the pathogen. Normally, primers are the regions that are bound and promote

the initiation of sequencing; since these regions are conserved within the same organism, the same primers can be utilized to identify that organism. However, the modification of the primers would produce a strain of pathogen that the normal primers would not adhere to, making it increasingly difficult to identify the pathogen used in an attack. The final goal through genetic modification was to develop strains which were impervious to medical treatment (Davis, 1999). Today, there are strains of *Bacillus anthracis* (anthrax), *Bacillus mallei* (glanders), *Bacillus pseudomallei* (melioidosis), *Yersinia Pestis* (plague), and *Francisella tularensis* (tularemia) that are resistant to normal methods of treatment (Cote, 2020).

Modern Genetics Allows Creation of Chimeric Viruses

Chimera viruses are viruses containing nucleic acids from two or more different organisms, the impact of this being that a virus typically attributed to infecting one species now has the ability to have a high affinity for another species. Genetic engineering has allowed viruses originally classified as Category 3 bioagents to be elevated to Category 1 status. An example of this is the *SARS-CoV-2* virus which seems to have been originally the RaTG13 bat CoV virus (which did not have a high affinity for human receptors) but through a series of recombination events obtained spike proteins with a high specificity for human ACE2 receptors. This concept of chimera viruses is not new to the scientific community. The Soviet Union's Biopreparat in the 1980's and 1990's instituted the "Chimera Project" which sought to uncover a method to combine two different viruses to make a more potent bioweapon against humans by increasing the affinity to human receptors (Alibek, 1999).

Additionally, laboratories in China – specifically the Wuhan laboratories – have been researching chimera coronaviruses since 2007 when the laboratory successfully transferred the spike proteins from the human *SARS-CoV* virus to segments of bat CoV genetic backbones. This

granted the segmented portions of the bat virus the ability to bind human ACE2 receptors via the transferred viral proteins. The University of North Carolina in 2008 modeled these same chimeric modifications in a live version of the virus by adding the human *SARS-CoV* spike proteins to a live version of the bat viral backbone (as opposed to the segments of the virus tested by Wuhan in 2007). Thus, a live *SARS-CoV* chimera was manufactured. In 2015, a paper was published demonstrating Wuhan's collaboration with the University of North Carolina and subsequently detailing the successful formation of additional chimeric CoV viruses (Segreto, 2020). These genetic advancements have created a whole new playing field for biowarfare, and in order to effectively analyze the current trajectory of modern bioweapons programs, it is essential to first understand from a historical perspective the developmental progression of offensive biological weapons programs.

Historical Analysis of International Bioweapons Program Development

Utilized for assassinations from the very beginnings of documented history, poisons have long posed a threat to human life; however, comprehensive biological weapons programs did not pose a substantial threat until Robert Koch and Louis Pasteur provided the scientific community with a foundation in microbiology in the 1800's. This background in microbiology furnished the knowledge necessary to begin honing biological agents for large scale military usage (Frischknecht, 2003). The World Wars provided the appropriate setting for scientifically advanced nations to test biological agents on a large-scale platform.

While Germany was the first nation to deploy weapons of mass destruction, they failed in their attempts to instigate a widespread infection of livestock and food supplies with anthrax during World War I as their attacks produced minimal effect on the targets. The usage of chemical warfare agents in World War I prompted the formation of the Geneva Protocol in 1925

which prohibited the release of both chemical and biological warfare agents during a war (Bernstein, 1987). However, this protocol did not prohibit the research and development of chemical and biological warfare agents, and thus did nothing to thwart the development of substantial biological and chemical warfare programs.

Soviet and Japanese Bioweapons Program Development

The first truly effective and comprehensive biowarfare programs were developed in the Soviet Union in 1928 and Japan in 1930 under the directions of Vladimir Lenin and Shiro Ishii, respectively. Shiro Ishii started his research in the Tokyo Army Medical School and utilized World War II as an opportunity to brutally test his developed pathogens on Chinese citizens and prisoners of war. At the height of the Japanese bioweapons program during this period, over 600 humans were killed annually in just one of the twenty-six test facilities from being cruelly subjected to human studies (Frischknecht, 2003).

Simultaneously, the Soviet Union was rapidly advancing their arsenal of bacterial and viral pathogens but had not yet established an effective delivery system for the pathogens. This changed at the conclusion of World War II when the Soviet military obtained Japanese biowarfare documents detailing the Japanese biological weapons models as well as two books from the United States written by Theodor Rosebury detailing the United States' plans for deploying biological agents (Leitenberg, 2014). These documents provided the Soviet Union with the necessary arsenal to effectively distribute biological agents in a large-scale attack and their research over the next several decades provided the foundation required for the formation of the Biopreparat – one of the largest bioweapons programs the world had ever seen (Leitenberg, 2014; Frischknecht, 2003).

American Bioweapons Program Development

During World War II, the Chemical Warfare Service in the United States began devoting limited quantities of its resources to the research of biological weapons, however, this research was widely kept secret and the President of the United States was unaware of its existence. It was not until Secretary of War Henry L. Stimson advised President Franklin D. Roosevelt of the advantages of a biological weapons program that one was officially created. This program was formed as the War Research Service in 1942 and was headed by George W. Merck – chemist and president of the Merck & Co. pharmaceutical company (Bernstein, 1987).

The formation of the War Research Service (WRS) permitted the expansion of the biological weapons program in the Chemical Warfare Service (CWS), as funds from the WRS were reallocated to the projects at the CWS. Increased funding for the CWS allowed the programs to extend into research facilities at Fort Detrick, Maryland. By the end of 1943, the CWS was rapidly developing a 500-pound anthrax bomb that contained smaller bomblets intended to disperse fatal pulmonary anthrax (Bernstein, 1987). The predominant focus of the program was weaponizing the anthrax and botulinum toxins given these agents tended to exhibit resilient characteristics, quickly proliferated, and had a very short incubation period. However, the U.S. bioweapons program received massive backlash from the general public during the anti-war movement in the Vietnam War era, and President Nixon vowed to abolish both biological and chemical weapons programs in 1969.

Biological Weapons Convention and Biopreparat

In 1972, the Biological Weapons Convention was formed. This convention sought to institute an international treaty signed by the majority of nations around the world (including the United States, the Soviet Union, and China). The goal of the treaty was to prohibit the research

and development of pathogens that could be utilized in an offensive attack as well as prohibit the stockpiling of biological weapons. Despite the formation of the treaty, countries remained in violation of the BWC – one of the most prominent examples being the Soviet Union and the formation of their Biopreparat (Frischknecht, 2003). The Biopreparat was developed just after the initiation of the BWC in 1972 and at the height of its activity, the Biopreparat employed over 50,000 people (Frischknecht, 2003). Suspicious activities in the Soviet Union such as the release and subsequent outbreak of inhalation anthrax from a military weapons facility in Sverdlovsk in 1979 alerted the United States to the fact that the Soviets were continuing to strengthen their biological arsenal. This prompted the United States to respond by expanding the American biological weapons arsenal as well (Bernstein, 1987).

Anthrax: Historical Case Study of One of the Oldest Biowarfare Agents

One of the very first pathogens utilized in biowarfare, *Bacillus anthracis* – more commonly known as anthrax – has plagued the field of agriculture through the infection and subsequent decimation of livestock populations. Generally, anthrax spores contaminate the feed and are ingested by cattle or elk, resulting in death of the animals. However, the development of modern biological weaponry programs discovered that anthrax is also extremely potent to humans if inhaled. There are three main forms of anthrax exposure: ingestion, inhalation/pulmonary, and cutaneous. For humans, the deadliest form of exposure is through inhalation as the pulmonary anthrax results in lesions on the lungs, necrosis of the tissue, and eventually suffocation leading to death. Since anthrax has long been utilized as a biological warfare agent, a detailed study of this historical agent provides the foundation for researching novel and emerging biowarfare agents.

Cellular and Genetic Structure of Anthrax

Gram positive and rod-shaped, the *Bacillus anthracis* organism is a single-celled, spore-forming bacteria that contains a single chromosome accompanied by two virulence plasmids. Studies have revealed that the chromosome does little to impact the toxicity of anthrax and has concluded that without the presence of the pXO1 and pXO2 plasmids, anthrax would not be toxic to humans. The pXO1 and pXO2 plasmids provide the genetic code responsible for the formation of the poly-D-glutamyl capsule which shields the organism from the immune system of the host it is invading as well as codes for the three factors that complex in order to form the toxins responsible for necrosis of tissues (Laws, 2016).

Anthrax Invasion of Host Cells

pXO2 codes for a poly-D-glutamyl capsule that allows the anthrax organism to evade phagocytotic responses by B cells, dendritic cells, and macrophages in the host immune system as well as making the bacteria impervious to bactericidal serums produced by the host (Splino, 2005). Once the anthrax organism successfully invades the host, the pXO1 plasmid produces the protective antigen, edema factor, and the lethal factor. Independently, these three factors have little impact on the host, but once the edema factor and the lethal factor couple with the protective antigen, they form the edema toxin and the lethal toxin, respectively (Hepburn, 2007).

Protective Antigen Forms Pore Allowing Anthrax Toxins into Host Cells

The protective antigen facilitates the movement of the edema toxin and the lethal toxin by associating into heptamers that form a pore in the membrane of the host cells, allowing the transport of the edema toxin and lethal toxin. Upon coming into contact with the host cells, the 83 kDa protective antigen binds to the anthrax receptors known as tumor endothelial marker 8 (TEM8, ANTXR1) and capillary morphogenesis gene 2 (CMG2, ANTXR2) (Friebe, 2016).

Normal functions of these receptors are not widely understood, however, the binding of the protective antigen to these receptors not only initiates the oligomerization of the protective antigen components, but it also dictates where the proteins that were internalized through endocytosis are released within the cell. Binding of the receptors initiates cleavage of the 83 kDa protective antigen by furin-like proteases to a 63 kDa form of the protective antigen. Upon being cleaved, the 63 kDa protective antigen will oligomerize and form a heptamer complex on the surface of the cell – only the cleaved form of the protective antigen is capable of oligomerizing (Shlyakhov, 1997).

In order for the protective antigen to facilitate the movement of the lethal factor and the edema factor it must be in its clustered, heptamer form. Once clustered, the lethal factor and edema factor can bind to the protective antigen. Clathrin-mediated endocytosis internalizes the entire hetero-oligomeric toxin-receptor complex using an endosome. The low pH within the endosome results in a conformational change of the protective antigen heptamer, which forms a pore in the membrane of the endosome. This permits the movement of the lethal toxin and the edema toxin into the cytoplasm of the host cell (Shlyakhov, 1997).

Lethal Toxin and Edema Toxin Cellular Functions

The lethal antigen is a zinc metalloprotease that cleaves the N-termini of mitogen activated protein kinases kinases (MAPKKs) belonging to the MEK family. The MEK kinases are essential for the regulation of the ERK, JNK, and p83 pathways responsible for the regulatory functions of the cell pertaining to cell function, growth, and stress response. Deactivation or inhibition of these pathways through the cleavage of the MAPKKs results in a major disturbance of normal cell activity (Shlyakhov, 1997).

The edema factor is a highly efficient calmodulin-dependent adenylyl cyclase that greatly increases the turnover of AMP to cAMP, which can act as a secondary messenger that leads to cell death. Both of these factors inhibit an early immune response, allowing the invasion to progress into later stages. The later stages of the anthrax toxins lead to tissue necrosis and cell death; prolonged duration of infection increases the damage to tissues and eventually leads to the death of the host (Shlyakhov, 1997).

Anthrax in Biological Outbreaks and Bioterrorist Attacks

Anthrax remains one of the longest offensively utilized biological agents in history as it was one of the first pathogens tested when nations were initiating their BW programs in the early 1900's. Two specific attacks and outbreaks in recent decades have highlighted just how potent anthrax is and how detrimental it is to human populations. In 1979 a military research facility specializing in microbiology in Sverdlovsk, Russia experienced a containment breach and leaked inhalation anthrax through the vents of the facility. The anthrax spores were carried through the air into the town, infecting the citizens within the city and resulting in the deaths of approximately 66 civilians. In 1998, tissue samples from 11 of the victims of the Sverdlovsk outbreak were obtained and it was determined through sequencing the DNA that there were four different genetic variants of the anthrax in the outbreak. Natural outbreaks of anthrax would have resulted in a single strain of the pathogen being recovered, thus the presence of four variants allowed researchers to conclude that on some level, genetic engineering of anthrax was occurring within the military complex (Ainscough, 2002).

Additionally, in 2001, the United States experienced a series of bioterrorist attacks when a powdered form of anthrax was distributed through the U.S. postal service just after 9/11. The series of attacks led to the deaths of five individuals, the infection of 17, and targeted several

high-profile persons such as Senator Patrick Leahy and Congressman Rush Holt. The Federal Bureau of Investigation (FBI) with the help of the U.S. Army Medical Research Facility ultimately concluded that the anthrax strain within the envelopes contained the Ames strain of anthrax – the reference strain of anthrax – and was not genetically altered. The FBI also concluded that the different envelopes contained varying purities of anthrax, suggesting that the individual or group responsible for the attack had been culturing and successfully developing several aliquots of the RMR-1029 anthrax culture (Majidi, 2008).

Mentioned previously, when hostile nations such as the Soviet Union sought to genetically modify anthrax the goal was to engineer a strain that would bypass defense systems, persist in the environment, be undetectable by diagnostic testing, and be untreatable by existing medical antibiotics. The motive behind all these modifications were to ensure that when deployed the pathogen would have the highest infection rates against human targets. This is a common theme in the genetic engineering of bioweapons – to ensure that it has maximum effect by increasing specificity against the target.

SARS-CoV-2 **and its Genetic Variances**

SARS-CoV-2 – also known as COVID-19 and the Coronavirus – has resulted in a pandemic over the last two years that has wreaked havoc on the entire globe not only through a public health crisis but also by massively impacting economies. The effort to effectively combat *SARS-CoV-2* has prompted scientists in government, industry, and academia to band together and research the infection mechanism of Covid-19, its pathogenicity, and the potential for vaccines to protect vulnerable populations. This research has demonstrated that there remain two unique attributes to *SARS-CoV-2* that have not been previously seen in other Sars-CoV strains. In order

to appreciate the significance of these cellular modifications it remains essential that the infection mechanism of *SARS-CoV-2* is understood.

Cellular and Genetic Structure of *SARS-CoV-2*

SARS-CoV-2 is a single stranded positive RNA virus approximately 29.9 kilo-base pairs long belonging to the Coronaviriae family in the betacorona 2B lineage. It is made of four structural proteins beginning with the nucleocapsid proteins which make up the capsid surrounding the viral genetic material (Wang, 2020). The envelope is made of the three additional structural proteins: the membrane proteins, spike proteins, and envelope proteins. Beyond the structural proteins *SARS-CoV-2* produces 16 non-structural proteins. Probably one of the most essential proteins for the virus, the spike protein consists of two non-covalently bound subunits referred to as the S1 and S2 subunits. The S1 subunit contains the N-terminal domain and the receptor binding domain (RBD), which is essential for binding the human ACE2 receptor. The S2 subunit is made of a fusion peptide, heptad repeat 1, central helix, connector domain, heptad repeat 2, transmembrane domain, and a cytoplasmic tail (*Figure 1*). This subunit is responsible for fusing to the membranes of the host cells for binding via the RBD. Between the S1 and S2 subunits there is a cleavage site that is required for activation of the proteins via an irreversible conformational change promoting the active binding state (Wang, 2020). In the *SARS-CoV-2* virus, the virus is activated through cleavage by the protease enzyme furin, an ubiquitous enzyme in humans.

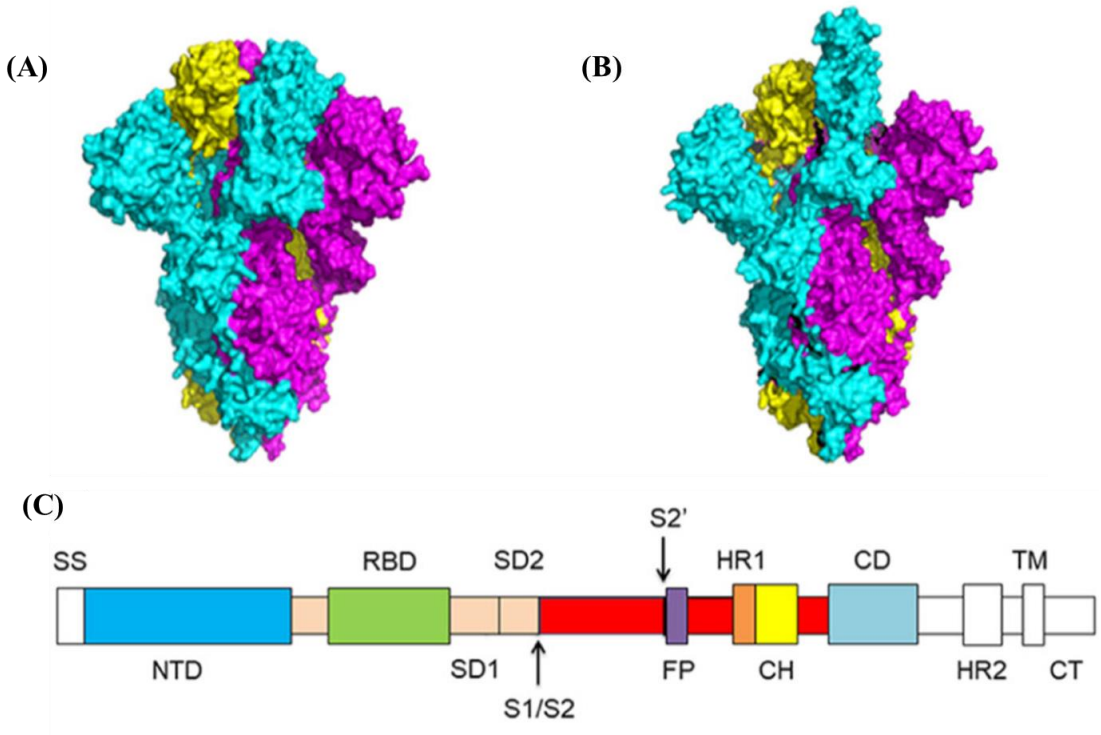


Figure 1. Model of the S protein subunits for S1 and S2. (A) Shows the S protein in its closed conformation and (B) shows the S1 protein in the upright position so that it is able to bind the ACE2 receptor. (C) Displays the different domains of the S protein in the following order: SS – single sequence, NTD – N-Terminal Domain, RBD – Receptor Binding Domain, SD1 – Subdomain 1, SD2 – Subdomain 2, S1/S2 – S1/S2 cleavage site, S2' – S2' protease cleavage site, FP – fusion peptide, HR1 – heptad repeat 1, CH – central helix, CD – connector domain, HR2 – heptad repeat 2, TM – transmembrane domain, CT – cytoplasmic tail. Arrows show where the protease cleavage sites are. Figure adapted from (Wang, 2020).

Cellular Infection Mechanism of *SARS-CoV-2*

Binding a receptor is the first step that *SARS-CoV-2* must take in order to gain entry into human host cells and initiate the infection process. Research has demonstrated that the *SARS-CoV-2* virus binds the human angiotensin-converting enzyme 2 receptor, also known as ACE2

through viral spike protein contact with the receptor (Harrison, 2020). The spike protein found on the surface of *SARS-CoV-2* is heavily glycosylated and is classified as a trimeric class I fusion protein. Structure of this spike protein includes three S1 protein receptor heads mounted on a S2 protein stalk. These S1 proteins display two different orientations that dictate whether the spike protein is able to bind a host cell or not. When the S1 protein is in the down position (*Figure 2A*), the virus is not able to bind the host cell (*Figure 2B*), which allows it to maintain a state that aids in its evasion of the host immune system. S1 protein upward orientation (*Figure 2C*) results in a spike protein activated to bind the ACE2 receptor on the surface of the host cell (Shang, 2020). The RBD on the surface of the virus is dependent on the targeted cells producing TMPRSS2 and cathepsin L in order for the S1 protein to be activated and primed for a binding event with a receptor (Harrison, 2020).

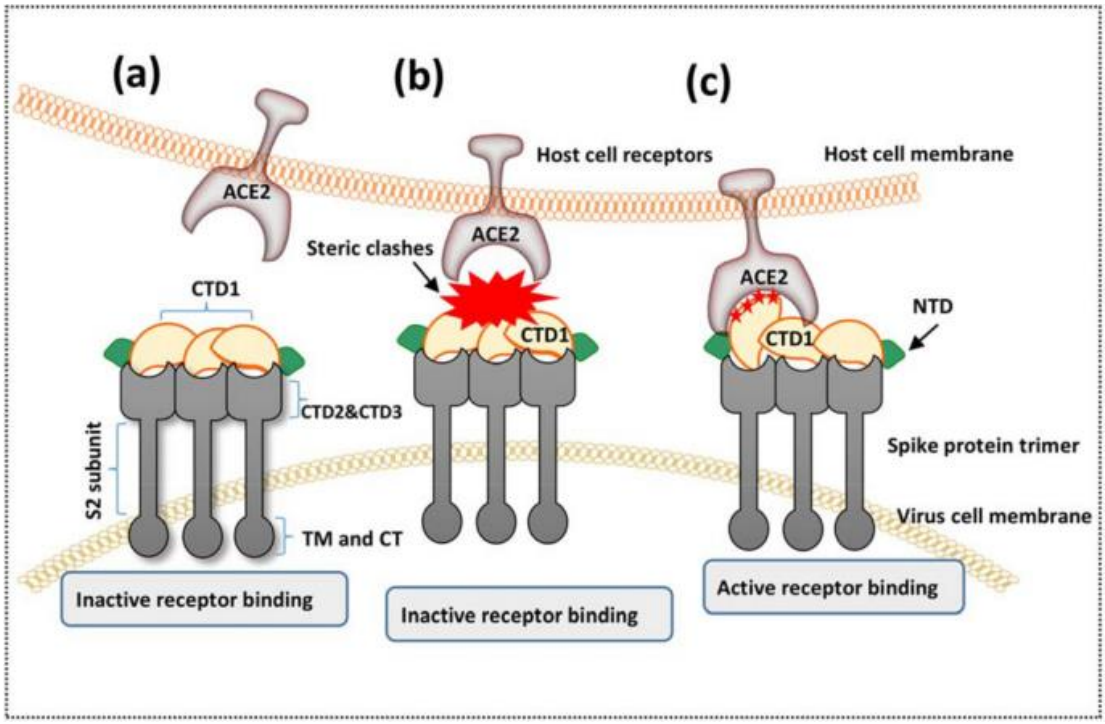


Figure 2. Molecular conformations of the S1/S2 spike protein and subsequent binding effectiveness. **(a)** Displays the S1 subunit in the downward position which in turn prevents binding to the human ACE2 receptor as shown in **(b)**. **(c)** shows the S1 protein in the upward position which allows for binding to the ACE2 receptor, leading to entry and to the infection of the host cells. Figure modified from (Yesudhas, 2021).

Receptor Binding Domain has High Affinity for Human ACE2 Receptor

Studies have demonstrated that the point mutations on the receptor binding domain (RBD) of the *SARS-CoV-2* virus compared to other Sars-CoV viruses creates a higher binding affinity between *SARS-CoV-2* and the human ACE2 receptor as the altered structure of the spike protein results in closer contact between the spike protein and its receptor (Harrison, 2020). Current scientific theory is that the *SARS-CoV-2* virus contains a RaTG13 viral backbone – the CoV strain specific to bats. While the RaTG13 does have the potential to bind the human ACE2

receptor, this occurs at a much lower affinity and lower strength than the *SARS-CoV-2* virus (Liu, 2021). Additionally, *SARS-CoV-2* displays a higher binding affinity to the ACE2 receptor than seen with the Sars-CoV virus. While this is not necessarily indicative of a stronger bond (in fact, research has shown that the Sars-CoV virus has a stronger bond to the ACE2 receptor), it is more likely to bind the ACE2 receptor due to its conformational variation (Harrison, 2020). The Harrison research group indicates that the difference in binding strengths between *SARS-CoV-2* and Sars-CoV virus could be the result of the two different orientations manifested by the S1 proteins on the *SARS-CoV-2* spike protein, meaning that the downward positioning of the S1 protein makes it less accessible.

Novel Furin Cleavage Site on SARS-CoV-2 Creates Potency in Additional Human Tissues

However, a new binding site at the S1/S2 junction known as the furin cleavage site accounts for the decreased accessibility from the downward positioning of the S1 spike protein as it also enhances the affinity of the virus for human cells. The furin cleavage site ensures that the virus is less dependent on host cell machinery, and it allows the virus access to varying tissue cells that it would not normally have access to. Coronaviruses normally rely on the presence of host cells expressing TMPRSS2; when TMPRSS2 and host enzyme cathepsin L are expressed the ACE2 receptor permits entry of the virus into the host cells. However, *SARS-CoV-2* experienced an addition of a furin cleavage site, enabling the virus to be preactivated through cleavage and able to bypass the requirement for TMPRSS2 and cathepsin L (Harrison, 2020).

Receptor Binding Domain Point Mutations of *SARS-CoV-2*

China and the United States have long been researching the possibility of creating cross-species viruses – also referred to as chimeric viruses – that would allow a virus characteristic to one species to jump from species to species, increasing the rate of infection. Generally, the

coronavirus has been seen presented in bats, and is typically referred to as the RaTG13 strain. Since 2007, the Wuhan Institute of Virology has been studying the potential for bat viruses to be genetically modified in such a way that would allow these pathogens to infect humans (Segreto, 2020). Specifically, the goal was to modify the spike protein in such a way that it removed the bat proteins and attach new proteins to the surface of the spike proteins so that the virus's receptor binding domains would have a high affinity for human receptors. RaTG13 was not previously observed infecting humans, rather the purpose of this study was to modify the virus in a manner that would allow its spikes to bind human receptors. The Wuhan research team was able to accomplish this goal and created several new strains of the bat virus that were capable of infecting humans.

Throughout the research into the origin of *SARS-CoV-2* and the modifications of the receptor binding domain proteins, it was noticed that while the backbone of the virus was nearly identical to that of the RaTG13 virus with a reported similarity of 96.2% (*Figure 3*), the spike proteins were not as similar between the strains (Lv, 2020; Flores, 2020). This prompted the search for a natural intermediary host that would have facilitated the movement of the virus from bats through the intermediary host to humans. Research found that the RBDs on the *SARS-CoV-2* were almost identical to the spike proteins found on the pangolin strain (referred to as the MP789 strain) presenting a 97.2% amino acid similarity (*Figure 4*) between the two strains while the amino acid similarity between RaTG13 and MP789 was only 89.2% (Han, 2020).

Binding studies were conducted analyzing the affinities for the *SARS-CoV-2* RBD for cellular receptors on bat, pangolin, and human receptors. It was determined that the RBD on *SARS-CoV-2* maintained the highest binding affinity for human ACE2 receptors over pangolin receptors. The high affinity for humans again points to highly specific changes in the RBD spike

proteins that granted the virus the ability to have a significant affinity to human receptors compared to those RBDs found in the RaTG13 and the MP789 strains. Additionally, it was observed that the RaTG13 does not bind to the pangolin ACE2 receptors, which is the equivalent receptor that the *SARS-CoV-2* binds to in humans (Segreto, 2020).

(A)

(B) 96.2 % Whole Genome Similarity to SARS-CoV-2

Figure 3. Chinese horseshoe bat responsible for RaTG13 virus and genetic similarity to SARS-CoV-2. **(A)** Depicts a Chinese horseshoe bat that is the primary host for RaTG13 strain of the coronavirus. **(B)** Shows there is a high whole genomic similarity between the RaTG13 virus and *Sars-CoV-2* at 96.2%. Figure modified from (Yong, 2013).

(A)

(B)

Figure 4. Chinese pangolin responsible for MP789 virus and its genomic similarity to SARS-CoV-2. **(A)** Depicts a Chinese pangolin (an anteater like animal) that is the primary host for the MP789 strain of the coronavirus. **(B)** Shows that there is a high similarity between the RBD of MP789 to the RBD of *SARS-CoV-2*. Figure modified from the Zoological Society of London.

Table 1.

Percent Similarity of Receptor Binding Domain (RBD) Genomic Region to the Pangolin MP789.

RBD Nucleotide Similarity to Pangolin *MP789*		
	RaTG13	*SARS - CoV - 2*
RBD of MP789	89. 2 %	**97.2%**

Note. Data was taken from (Han, 2020).

Table 2.

Percent Similarity of Whole Genome Sequences to *SARS-CoV-2* Virus.

Whole Genome Nucleotide Similarity to *SARS - CoV - 2*		
	RaTG13	*MP789*
SARS - CoV - 2	**96.20%**	85 - 92 %

Note: Data was taken from (Flores, 2020).

Evidence of Man-Made Chimeric Creation

Lack of Pangolin Population Limits Opportunity for Natural Recombination Event

Researchers have proposed that the origin of the *SARS-CoV-2* virus – which presents with a RaTG13 genetic backbone and MP789 spike proteins – could have originated through natural recombination events between the RaTG13 strain and the MP789 strain. Pangolin population and habitat studies have recently revealed that the pangolin population in China has greatly declined over the last several decades to the point that they are considered almost extinct in some regions due to habitation loss and the marketing of these animals for their scales.

In the past, the Hubei province of China has recorded established populations of pangolins in the northwestern region, specifically in the Shiyan City as well as the Danjiangkou and Fangxian counties (Zhang, 2022). However, these counties are located in the northwestern portion of Hubei, and Wuhan – the place of origin for *Sars-CoV-2* – is situated in the southeastern portion of the province approximately 250 miles away from these locations. While from a genetic perspective the proposed recombination events are possible in a natural setting, the lack of a viable pangolin population that would be required in the Wuhan region to facilitate this type of recombination event makes it highly unlikely that this event occurred.

Addition of Furin Cleavage Site on SARS-CoV-2

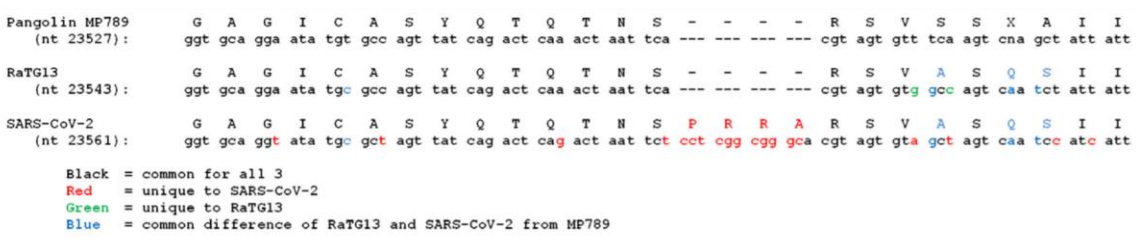
Analysis of the activation method of the *SARS-CoV-2* spike proteins revealed a furin cleavage site that has not been previously seen on other coronavirus strains. The presence of the furin cleavage site is significant given furin is ubiquitous in all human tissues, which decreases the virus's reliance on host cell machinery for activation and permits it to enter tissues that it would not typically have access to. Activation of the spike proteins via the presence of furin is what grants the *SARS-CoV-2* virus the ability to infect human lung tissue as well as initiate a systemic infection throughout the body.

The genetic code responsible for the furin cleavage site was the 12 nucleic acid sequence 5'-T-CCT-CGG-CGG-GC - 3' that was inserted at the S1/S2 junction (*Figure 5A)*. This 12 nucleic acid sequence is not present in any other Sarbecoviruses, and bioinformatic analysis demonstrated that this addition of the nucleotides had to have come by means of an insertion – not through a frameshift or deletion (Diegen). What researchers have found unusual about this insertion is that two arginine residues are coded for by CGGCGG which is not the predominant codon for arginine in either the *SARS-CoV-2* or the RaTG13 strains. In fact, approximately only 5% of arginine amino acid residues are coded for with the CGG sequence, and yet in this insertion two back-to-back CGG additions are observed (Segreto, 2020). Thus, this poses the question as to why in a 12 nucleic acid insertion, 6 of those 12 nucleotides are in an arrangement that is rare in the genomic sequence of coronaviruses.

Closer examination of an amino acid codon chart reveals that the amino acid arginine is one of two amino acids that can be coded for by six different codons. The codon usage table establishes that in nature, the CGG codon is used for arginine only 11% of the time (*Figure 5B*). Nature uses what is readily available to it, which means that the tendency would be to utilize the

more predominant codons such as CGT and CTC which are both utilized 36% of the time. It seems unlikely that nature would repeat a codon only experienced 11% of the time twice in a row in an insertion mutation. In fact, repeated patterns of codons tend to be attributed to man-made cloning techniques especially when utilizing a rare codon sequence. Due to the low probability of the CGG codon occurring multiple times in nature, this 5' – CGG-CGG – 3' repeat points to the insertion sequence being constructed and manually inserted into the *SARS-CoV-2* virus.

(A)



(B)

CGT	R	0.36	20.0	73197
CGC	R	0.36	19.7	72212
CGA	R	0.07	3.8	13844
CGG	R	0.11	5.9	21552
AGA	R	0.07	3.6	13152
AGG	R	0.04	2.1	7607

Figure 5. Alignment of MP789, RaTG13, and SARS-CoV-2 reveals a 12 nucleic acid insertion in the SARS-CoV-2 virus. **(A)** This figure displays the 12 nucleic acid insertion in the *SARS-CoV-2* virus showing that this sequence was not present in the MP789 virus nor the RaTG13 virus. It illustrates the repeated CGG codon for arginine. **(B)** This figure is an excerpt from a codon usage table and reveals that the CGG codon for arginine is only seen used in nature 11% of the time. The more predominant codons for arginine are the CGT and CGC codons which are both observed 36% of the time in nature. Figure (A) modified from (Segreto, 2021) and Figure (B) modified from (NCBI – GenBank, 2007).

Implication of Genetic Alterations

Ultimately, new genetic modifications displayed in *SARS-CoV-2* demonstrate that the virus experienced a series of mutations that granted it a higher affinity for binding human ACE2 receptors, creating a chimeric virus. Not only does the spike protein have a great affinity for binding and infecting human cells, the addition of the furin cleavage site permits the virus to now enter and infect a myriad of human tissues that it did not previously have access to, including lung tissue. This modification results in the virus now having the capability to produce a systemic infection within patients. Additionally, viral spike proteins are less reliant on having the proper human cell machinery present for activation as furin is naturally occurring in all human tissues. Lack of a pangolin population in the Wuhan area seems to limit the probability of a naturally occurring recombination event between the RaTG13 strain and the MP789 strain to create a virus with the RaTG13 backbone and the MP789 spike proteins. This suggests the possibility of genetic manipulation.

The Common Denominator: Furin Cleavage

Furin is a serine protease that is ubiquitous in all human tissues. Disorders involving this protease have been linked to the development of tumors and cancer, and given its production by human tissues, it is commonly exploited by viruses and bacterial pathogens as a means for activation and entrance into host cells (Braun, 2019). In many cases with viruses and bacterial pathogens, the furin cleavage for activation is not a requirement but rather something that makes the pathogen much more infectious. Historically utilized as an offensive bioagent, anthrax contains a furin cleavage site that allows it to activate its toxins, increasing the toxicity of the bacteria. Furin successfully cleaves and activates the protective antigen, promoting the entry of

the pathogen into the host cell and the subsequent formation of the edema toxin and the lethal toxin.

It is interesting that the historically utilized bioagent anthrax contains a furin cleavage site and the *SARS-CoV-2* virus had a novel insertion that produced the furin cleavage site resulting in a more infectious strain of the coronavirus. The furin cleavage site is not required for viral entry and infection of human cells, but it grants the *SARS-CoV-2* virus access to lung tissue and ultimately makes it more potent against humans as it is able to lead to a systemic infection. While this could be a naturally occurring insertion, the precise location of the insertion coupled with the CGG codon for arginine and the RBDs with high binding affinity for human ACE2 receptors seems to indicate an intent to engineer a more infectious coronavirus strain for humans by altering the coronavirus with these modifications. It further supports the need for continued investigation into the origin of the *SARS-CoV-2* virus and the possibility of its originating in a laboratory rather than a natural setting.

Bioterrorism Implications for the United States

If the *SARS-CoV-2* virus arose in a laboratory, it would have major biodefense and biosecurity implications for the United States. Firstly, the United States would need to address the fact that genetic engineering opens the door to a wide variety of new viruses and bacterial pathogens that can be genetically weaponized through modifications intended to increase the lethality within human populations. Whether or not the *SARS-CoV-2* virus was genetically engineered to be a bioweapon or not, it was a virus that was modified to increase human infection rates through the addition of RBD spike proteins specific to human ACE2 receptors. This should spark the question as to why China would need to research the potential to alter a virus previously inert to humans to a chimeric strain now capable of infecting humans to a higher

degree. If the *SARS-CoV-2* virus was genetically engineered, it would not be the first time China worked on this type of project as a chimeric coronavirus project was funded in Wuhan laboratories in 2007 in collaboration with the United States. This should also raise the question of whether China is now in violation of the biological weapons convention and if they are, how the United States should seek to respond.

Evolving technologies generate advanced delivery systems for bioagents permitting a more targeted release of an agent. The *SARS-CoV-2* virus does not appear to have any pinpointed dispersal target as it has resulted in a global pandemic; however, the genetic modifications within this virus should cause the United States to consider how other pathogens might be modified and coupled with a high-tech dispersal system to infect specific target bases. The last decade has seen the development of advanced unmanned aerial vehicles (UAVs) and subsequently the introduction of a new biowarfare dispersal system. These UAVs have the ability to fly under the radar of typical defense monitoring systems and can be outfitted with deployable bioweapons (Hoenig, 2014). Additionally, drones are already utilized to spray antibiotics on crops in an agricultural setting. In the same way the aerosol outbreak occurred through a ventilation system in Sverdlovsk, it would be feasible to outfit agricultural drones with a bioagent, allowing the dispersal of an aerosolized strain of virus or bacteria.

Ultimately, there are still many questions revolving around the origin of the *SARS-CoV-2* virus. Comparing this virus to a bacterial pathogen long utilized as a bioweapon demonstrates that the insertion of the furin cleavage site seems to suggest more than natural origins for this virus. This places the United States biodefense and biosecurity fields in a pivotal position as they have the opportunity to choose to learn from the outbreak of *SARS-CoV-2* and research how to

better safeguard against future genetic modifications of other bioagents, addressing the increasing threat of evolving dispersal systems.

Though genetic mutations and the lack of a pangolin population in Wuhan – as well as Wuhan's history of researching *SARS-CoV* virus's potential for chimeric mutations – suggests that the *SARS-CoV-2* virus may have laboratory origins. This paper sought to highlight the potential for the development of future offensive bioagents with similar chimeric features to those of *SARS-CoV-2*. Some would argue that the reason scientists need to make chimeric viruses is so the medical community can be adequately equipped for potential outbreaks due to naturally arising chimeric infections through recombination events. However, the heightened specificity and affinity for the ACE2 receptors noted in the coronavirus and the continued research of chimeric viruses in laboratories escalates the risk of a biocontainment breach resulting in a pandemic. This should promote increased funding for the United States' biodefense programs in order to generate solutions to mitigate the fallout from attacks with chimeric viruses. The Unites States can effectively prepare for a chimeric attack with solutions ranging from the creation of rapid tests to more advanced detection systems that can efficiently identify the genomic components of viruses. Ultimately, development of viruses with chimeric characteristics are becoming increasingly prevalent as evident with modifications present in *SARS-CoV-2,* and thus it remains imperative for American biodefense and biosecurity that the United States acknowledge that chimeric viruses are the bioweapons of the future.

References

Ainscough, M. (2002). Next Generation Bioweapons: The Technology of Genetic Engineering Applied to Biowarfare and Bioterrorism. *United States Air Force Counterproliferation Center*, 14. https://purl.fdlp.gov/GPO/LPS105199.

Alibek, K. (1999). A Bio Nightmare: Biohazard. *SAGE Journals*, 55 (4), pp. 69-71. https://doi.org/10.2968/055004019.

Bernstein, B. (1987). The Birth of the U.S. Biological-Warfare Program. *Scientific American*, 256(6), pp. 116-121. https://www.jstor.org/stable/10.2307/24979407.

Braun, E. & Sauter, D. (2019). Furin-mediated Protein Processing in Infectious Diseases and Cancer. *Clinical and Translational Immunology*, 8, e1073, 10.1049/esej:19950205

Centers for Disease Control and Prevention. (2018). Bioterrorism Agents/Diseases. *Emergency Preparedness & Response*. https://emergency.cdc.gov/agent/agentlist-category.asp.

Cote, C., Irma, B., Hunter, M., Shoe, J., Klimko, C., Panchal, R., & Welkos, S. (2020). Microbial Pathogenesis. *Elsevier*, 142(2020), 104050. https://doi.org/10.1016/j.micpath.2020.104050.

Davis, C. (1999). Nuclear Blindness: An Overview of the Biological Weapons Programs of the Former Soviet Union and Iraq. *Johns Hopkins University Center for Civilian Biodefense Studies*, 5 (4), pp. 509 – 512.

Deigin, Y. & Segreto, R. (2021). *SARS-CoV-2*'s Claimed Natural Origin Is Undermined by Issues with Genome Sequences of Its Relative Strains. *Bioessays*, 43 (2021), pp. 1-8. https://doi.org/10.1002/bies.202100015.

Flores-Alanis, A., Sander-Miranda, L., Delgado, G., Cravioto, A., Morales-Espinosam, R. (2020). The Receptor Binding Domain of *SARS-CoV-2* Spike Protein Is the Result of an

Ancestral Recombination between the Bat-CoV RaTG13 and the Pangolin-CoV MP789. *BioMed Central Research Notes,* 13 (398). https://doi.org/10.1186/s13104-020-05242-8.

Friebe, S., van der Goot, F., & Bürgi, J. (2016). The Ins and Outs of Anthrax Toxin. *Toxins*, 8 (69), pp. 1-15. doi:10.3390/toxins8030069.

Han, G. (2020). Pangolins Harbor *SARS-CoV-2* Related Coronaviruses. *Trends in Microbiology*, 28 (7), pp. 515 – 517. doi: 10.1016/j.tim.2020.04.001

Harrison, A., Lin, T., & Wang, P. (2020). Mechanisms of *SARS-CoV-2* Transmission and Pathogenesis. *Trends in Immunology*, 41 (12), pp. 1100 – 1115. https://doi.org/10.1016/j.it.2020.10.004.

Hepburn, M., et. al. (2007). Immune Response to Two Different Dosing Schedules of the Anthrax Vaccine Precipitated (AVP) Vaccine. *Elsevier*, 25 (2007), pp. 6089 – 6097. doi:10.1016/j.vaccine.2007.05.018.

Hoenig, M. (2014). Hezbollah and the Use of Drones as a Weapon of Terrorism. *Public Interest Report*, 67 (2).

Laws, T., et. al. (2016). A Comparison of the Adaptive Immune Response between Recovered Anthrax Patients and Individuals Receiving Three Different Anthrax Vaccines. *PLoS ONE*, 11 (3), e0148713. DOI:10.1371/journal.pone.0148713.

Liu, K., et. al. (2021). Binding and Molecular Basis of the Bat Coronavirus RaTG13 Virus to ACE2 in Humans and Other Species. *Cell Press*, 184, pp. 3438 – 3451. https://doi.org/10.1016/j.cell.2021.05.031.

Lv, L., Li, G., Chem, J., Liang, X., and Li, Yudong. (2020). Comparative Genomic Analyses Reveal a Specific Mutation Pattern Between Human Coronavirus *SARS-CoV-2* and Bat-

CoV RaTG13. *Frontiers in Microbiology*, 11 (584717). https://doi.org/10.3389/fmicb.2020.584717.

Majidi, V. (2008). Science Briefing on the Anthrax Investigation. *Federal Bureau of Investigation*.

NCBI – GenBank. (2007). Codon Usage Table. *Department of Plant Gene Research*.

Segreto, R. & Deigin, Y. (2020). The Genetic Structure of *SARS-CoV-2* Does Not Rule Out a Laboratory Origin: *SARS-COV-2* Chimeric Structure and Furin Cleavage Site Might be the Result of Genetic Manipulation. *BioEssays Wiley*, 43 (3), e2000240. https://doi.org/10.1002/bies.202000240.

Shang, J., Wan, Y., Luo, C., Ye, G., Geng, Q., Auerbach, A., & Li, F. (2020). Cell Entry Mechanisms of *SARS-CoV-2*. *Proceedings of the National Academy of Sciences of the United States of America*, 117 (21), pp. 11727 – 11734. https://doi.org/10.1073/pnas.2003138117.

Shlyakhov, E., Rubinstein, E., & Novikov, I. (1997). Anthrax Post-Vaccinal Cell-Mediated immunity in Humans: Kinetics Pattern. *Elsevier*, 15 (6/7), pp. 631 – 636. doi: 10.1016/s0264-410x(96)00286-1.

Splino, M., Patocka, J., Prymula, R., & Chlibek, R. (2004). Anthrax Vaccines. *Annals of Saudi Medicine*, 25 (2), pp. 143 – 149. https://doi.org/10.5144/0256-4947.2005.143.

Thavaselvam, D. & Vkayaraghavan, R. (2021). Biological Warfare Agents. *Journal of Pharmacy and Bioallied Sciences*, 2 (3), pp. 179 – 188. doi: 10.4103/0975-7406.68499.

Tucker, J. (1998). Verification Provisions of the Chemical Weapons Convention and Their Relevance to the Biological Weapons Convention – Biological Weapons Proliferation:

Reasons for Concern, Courses of Action. *United States Department of Justice*, NCJ – 193965.

Wang, M., Zhao, R., Gao, L., Gao, X., Wang, D., & Cao, J. (2020). *SARS-CoV-2*: Structure, Biology, and Structure-Based Therapeutics Development. *Frontiers in Cellular and Infection Microbiology*, 10 (587269), https://doi.org/10.3389/fcimb.2020.587269.

Wrapp, D., et. al. (2020). Cryo-EM Structure of the 2019-nCoV Spike in the Prefusion Conformation. *Science*, 367 (6483), pp. 1260 – 1263. doi: 10.1126/science.abb2507

Yaeger, R. (1996). Protozoa: Structure, Classification, Growth, and Development. *Medical Microbiology 4th edition.* Galveston (TX): University of Texas Medical Branch at Galveston; Chapter 77. https://www.ncbi.nlm.nih.gov/books/NBK8325/.

Yesudhas, D., Srivastava, A., & Gromiha, M. M. (2021). COVID-19 outbreak: history, mechanism, transmission, structural studies and therapeutics. Infection, 49(2), 199–213. https://doi.org/10.1007/s15010-020-01516-2.

Yong, E. (2013). Holy SARS Origin, Batman!. *National Geographic*, https://www.nationalgeographic.com/science/article/holy-sars-origins-batman

Zhang, F., Wu, S., & Cen, P. (2022). The Past, Present and Future of the Pangolin in Mainland China. *Global Ecology and Conservation*, 33 (2022), e01995, https://doi.org/10.1016/j.gecco.2021.e01995.

Zoological Society of London. (2022). Chinese Pangolin. *Edge*. http://www.edgeofexistence.org/species/chinese-pangolin/.