Ancestral SARS-CoV-2 is not a natural virus because it cannot support sustained transmission in any animal species:

Ancestral SARS-CoV-2, with its metastable D614/Furin Cleavage Site phenotype, cannot be maintained outside of a laboratory environment

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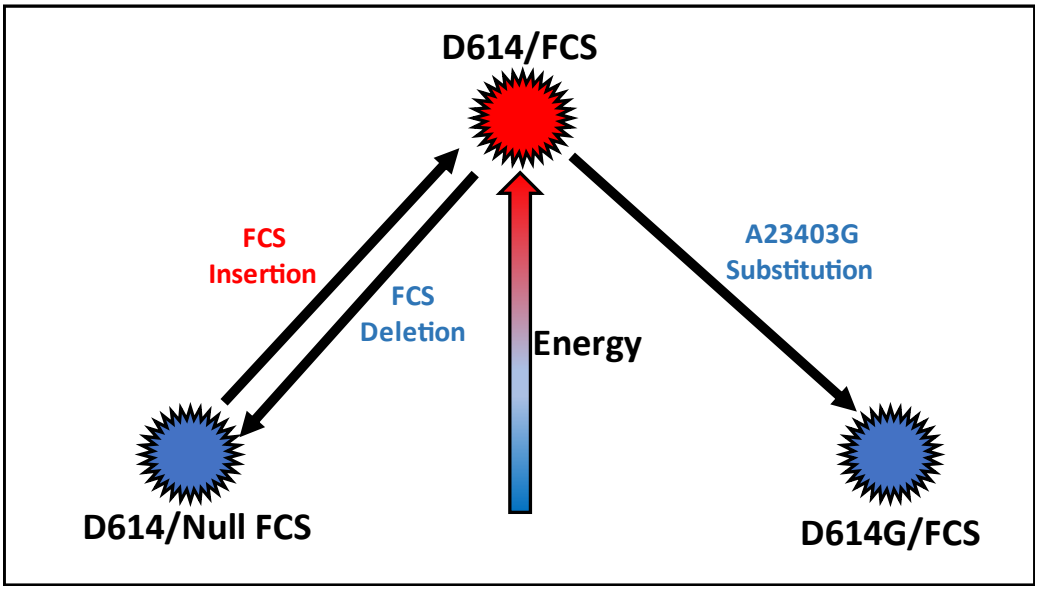
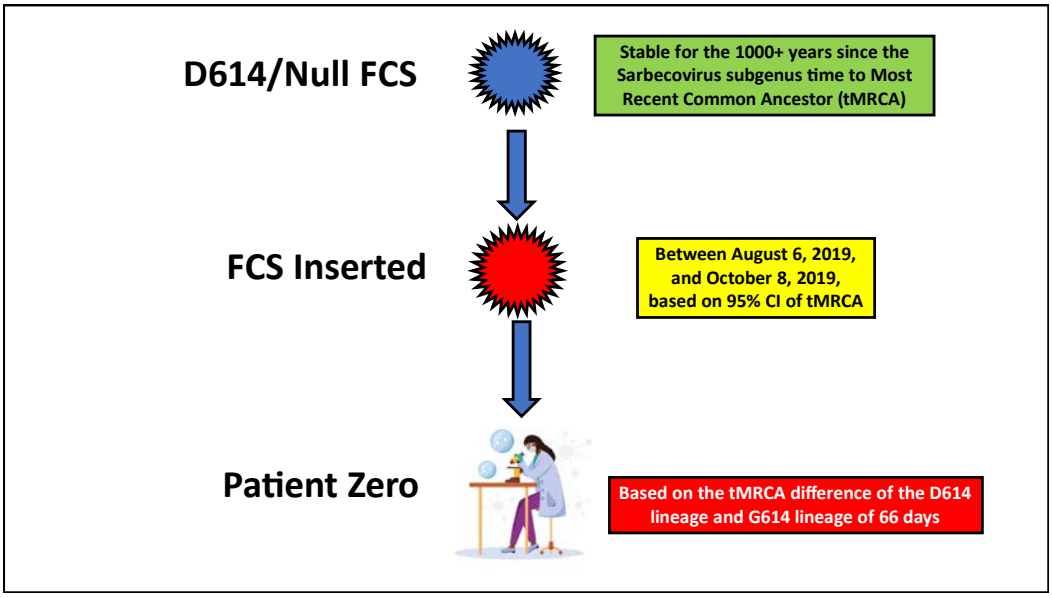
VISUAL ABSTRACT





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The FCS-cleaved Spike Protein of a D614 SARS-CoV-2 virus cuts the large Spike Protein into two separate, unattached peptides, the S1 and S2 portions of the protein. For the first time, the untethered S1 piece becomes unstable and leads to significant S1 shedding (perhaps assisted by antibody binding). This removes the RBD, rendering the virus inert and non-infective. This is an inherent instability property of the virus itself and has nothing to do with the host species. This precludes finding a host species that can effectively propagate and transmit the D614/FCS.

To compensate for this, the D614G substitution rapidly and independently evolved because it re-establishes S1 stability, restoring the S1 protein and its RBD to the viruses, and rendering them once again infective.

In laboratory cell culture, the inefficiency of a D614/FCS virus can be somewhat overcome by using large inoculums for propagation, but there is still a tendency for the G614 to outcompete the D614 virus variants.

The inherent instability of the first human SARS-CoV-2 sequences, with the D614/FCS phenotype, and its rapid conversion to D614G/FCS under all conditions of transmission outside of a laboratory, establishes that the ancestral SARS-CoV-2 is not a natural virus because it cannot support sustained transmission in any animal species and thus the pandemic was not a zoonotic spillover.

ABSTRACT

The origin of SARS-CoV-2 (CoV-2) remains unknown over 18 months after the COVID-19 pandemic began and because investigation inside China is currently impossible, only indirect methods of analysis are available. Here I use the metastable nature of the CoV-2 Spike Protein to infer the timing of the first human infection as well as the first zoonotic event. First, the progenitor of SARS-CoV-2 is determined to be D614/Null Furin Cleavage Site (FCS), based on the time to Most Recent Common Ancestor (tMRCA) for that phenotype within the *Sarbecoviruses* of 3500 BCE to 900 ADE. Next, I document that the D614/FCS phenotype is metastable under all experimental conditions. This is due to the shedding of the furin-cleaved S1 protein, generating non-infective particles, and is therefore an intrinsic viral property and is thus host species independent. The D614G substitution solves the S1 instability problem. Under all experimental conditions, the D614/FCS phenotype evolves into either the G614/FCS phenotype or reverts to the D614/Null FCS phenotype. In cell culture and animal-to-animal experiments these changes all begin in the first inoculation and become fixed within four passages. In human-to-human transmission, the G614/FCS phenotype appears within the first five mutations. Given that all initial patient CoV-2 infections were the D614/FCS phenotype and assuming the tMRCA for the COVID-19 pandemic is approximately November 13, 2019, the first introduction into a human can be no earlier than September 9, 2019. **Any earlier human infection would have already contained the G614/FCS phenotype in the first sequences at the beginning of the epidemic if the virus had been present in a non-human intermediate or reservoir host in the wild for any length of time before the beginning of the outbreak.** Similarly, at this time there has been no animal host identified in which the D614/FCS phenotype can be stably transmitted for more than a week; all infections rapidly evolve to the G614/FCS phenotype. Based on these findings, a zoonotic origin for COVID-19 requires a hypothesis that explains the highly adapted receptor binding domain present at the outset that supported human-to-human transmission, the insertion of the first FCS in any *Sarbecovirus* virus in more than 900 years, and the first animal-to-human transmission within literally days of that FCS insertion. The alternative and more parsimonious conclusion is that these steps were achieved by serial passage of a D614/Null FCS virus in non-human primates or humanized mice for ACE2 adaption, the laboratory insertion of the FCS, and an almost immediate laboratory-acquired infection, sometime after September 9, 2019.

INTRODUCTION

Within the *Sarbecovirus* subgenera, the D614/Null FCS phenotype is stable, first appearing over 900 years ago. However, upon introduction of the FCS and the cleavage of the Spike Protein in the Golgi by furin or TMPRSS2, the D614 phenotype makes the S1 protein fragment unstable. Up to 50% of all D614 virus particles have been found to lose the S1 fragment, making the particle noninfective. This property is a function of the virus Spike Protein and is therefore independent of any particular host species. Even the temperature stability of the virus is reduced in the D614/FCS phenotype.

During human-to-human transmission, multiple separate analyses document that the D614G variant arises within the first five mutations. In cells that are deficient in TMPRSS2, such as VERO cells, the S1 fragment is not generated and rather than the D614G variant arising the furin site is deleted, beginning in the first passage. In all animal species tested in which animal-to-animal transmission can be demonstrated the G614/FCS phenotype outperforms the D614/FCS phenotype.

In this paper I begin with the earliest SARS-CoV-2 virus sequence phenotype in the Spike Protein (SP) of 614D and 682RRAR685 (the furin cleavage site; FCS); henceforth the D614/FCS phenotype. This phenotype has been repeatedly demonstrated to be highly metastable, including in the human-to-human pandemic infections. As of December 22, 2024, for example, only 0.8% of the 17,122,506 sequences in the GISAID gene bank have the D614 phenotype.

RESULTS

D614/Null FCS has been a stable phenotype within the *Sarbecovirus* subgenera for between 900 and 5300 years

SARS-CoV-2, the virus responsible for COVID-19, is a member of the species *severe acute respiratory syndrome (SARS)-related coronavirus,* in the family *Coronaviridae*, subfamily *Orthocoronavirinae*, genus *Betacoronavirus*, subgenus *Sarbecovirus.* Jungreis et al. (2021) performed a codon level analysis of the SARS-related coronavirus species by comparing 44 SARS-related coronaviruses, including SARS-CoV-2, to determine protein coding and nucleotide (nt)-level evolutionary restraint within the species. The 44 coronaviruses were selected based on diversity of evolutionary distance, as measured by having on average *ca* three substitutions per four-fold degenerate codon nt site, a standard for other evolutionary studies.[2] The time to Most Recent Common Ancestor (tMRCA) for these 44 SARS-related *Sarbecoviruses* is approximately 1200 ADE, 900 years ago.[3] At the other extreme, the first appearance of the genus *Betacoronavirus*, the progenitor MRCA of the *Sarbecovirus* subgenera, has been estimated at 3300 BCE or about 5300 years ago.[4]

With respect to the D614 phenotype, it is present in all 44 SARS-related coronaviruses, including SARS-CoV-2, thus confirming it as an ancestral locus. In fact, the D614 residue (nt A23403) is a perfectly conserved nucleotide in a perfectly conserved amino acid in a perfectly conserved 11-amino-acid region.

In a review of 1,000 *Sarbecovirus* Spike Proteins (not including any SARS-CoV-2 sequences) 999 contained the D614 amino acid.[5] The only exception was a SARS-related coronavirus P2, collected in the US in 2004 by scientists at the J. Craig Ventner Institute (GenBank FJ882963.1), with a D614G, nt A>G, locus.

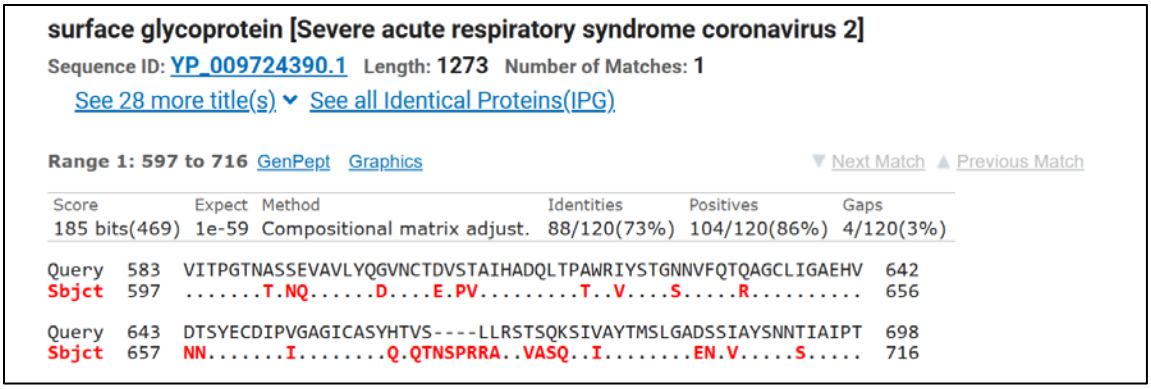
A comparison (below) of the 614/FCS region of the Spike Protein from P2 (Query) and SARS-CoV-2 (Sbjct) documents the D614G in P2 and the lack of a furin cleavage site, although the anchor cleavage dimer of the SARS-CoV-2 FCS, R/S, is also present in P2.

[2] Lindblad-Toh, K. et al. A high-resolution map of human evolutionary constraint using 29 mammals. *Nature* **478**, 476–482 (2011); Lin, M. F. et al. Revisiting the protein-coding gene catalog of Drosophila melanogaster using 12 fly genomes. *Genome Res.* **17**, 1823–1836 (2007).

[3] Boni, M.F., Lemey, P., Jiang, X. *et al.* Evolutionary origins of the SARS-CoV-2 sarbecovirus lineage responsible for the COVID-19 pandemic. *Nat Microbiol* **5,** 1408–1417 (2020). https://doi.org/10.1038/s41564-020-0771-4

[4] https://journals.asm.org/doi/10.1128/JVI.06540-11

[5] Bassa, B.; Olen, B. D614 Residue Belongs to a Highly Conserved Peptide Motif in *Sarbecovirus* Group and the D614G Mutation of SARS-CoV-2 Spike Protein Appeared Once in SARS-CoV. *Preprints* **2020**, 2020070488 (doi: 10.20944/preprints202007.0488.v1).



SARS-CoV-2, of course, has the S1/S2 FCS phenotype. The Null FCS phenotype is conserved in the remaining 43 SARS-related coronaviruses studied and is also found in the complete collection of *Sarbecovirus* sequences available in GenBank (approximately 1200).[6] No *Sarbecovirus* has ever been identified with an S1/S2 polybasic cleavage site of any amino acid sequence.

These findings establish that the FCS insertion, whether natural or laboratory engineered, occurred on the D614 background. In addition, x-ray crystallography has shown a salt bridge between D614-R646 which was originally proposed to stabilize the S1 Spike Protein fragment following FCS cleavage. Since only Q646 was found in the 43 *Sarbecoviruses* I will hypothesis that the progenitor to which the FCS was inserted will have both the D614 as well as the R646 phenotype.

The positive selection for the D614G phenotype has been proposed to be related to the improved physical stability of the S1 Spike Protein fragment following FCS activation and is therefore host independent

The D614G reduces S1 shedding following FCS activation and increases the quantity of Spike Protein incorporated into virions by 3.4- and 5-fold, respectively.[7] Once the FCS has been cleaved this stability change of the S1is a property of the SARS-CoV-2 virus Spike Protein and is not dependent on other host enzymes or factors, thus the positive selection pressure for the D614G substitution should be universal and independent of the viral host, whether in a reservoir host, an intermediate host, or a human host.

Another manifestation of the host-independent stability of the D614G variant is its temperature stability.[8] A comparison of SARS-CoV-2 variants with the D614 mutation and G614 mutation after different periods of refrigeration (4°C) and freezing (-20°C) was conducted. The results showed that SARS-CoV-2 was more stable and infectious after storage at -20°C than at 4°C and the G614 variant was found to be more stable than the S-D614 variant. The spike protein of the

[6] No sarbecovirus has ever had an FCS.
[7] Zhang, Lizhou & Jackson, Cody & Mou, Huihui & Ojha, Amrita & Erumbi, Rangarajan & Izard, Tina & Farzan, Michael & Choe, Hyeryunc. (2020). The D614G mutation in the SARS-CoV-2 spike protein reduces S1 shedding and increases infectivity. bioRxiv : the preprint server for biology. 10.1101/2020.06.12.148726.
[8] Huang SY, Kung YA, Huang PN, Chang SY, Gong YN, Han YJ, Chiang HJ, Liu KT, Lee KM, Chang CY, Chang CC, Huang CG, Shih SR. Stability of SARS-CoV-2 Spike G614 Variant Surpasses That of the D614 Variant after Cold Storage. mSphere. 2021 Mar 31;6(2):e00104-21. doi: 10.1128/mSphere.00104-21. PMID: 33789940.

G614 variant had better binding ability with the ACE2 receptor than that of the D614 variant after storage at -20°C for up to 30 days.

D614G evolves rapidly during human-to-human transmission

One study of 448 curated sequences showed the tMRCA for the D614 phenotype was November 13, 2019, while the G614 phenotype tMRCA was January 18, 2020, just 66 days later, with the 95% CI of 36 to 99 days.[9] Given a SNV rate of 25.8 per year,[10] D614G arose within the first 2.6 SNVs (95% CI of 1.4-3.8).

The D614G phenotype is not simply the result of a founder mutation which became fixed. The following Text-Table identifies five GISAID sequences in which the first SNV in a Clade G and a Clade O virus was, in fact, D614G, demonstrating independent geotemporal appearances in these two Clades.

Clade	Location	Identifier	Collection Date
G	Sichuan, China	EPI_ISL_451345	24-Jan-20
G	Munich, Germany	EPI_ISL_406862	28-Jan-20
G	Shanghai, China	EPI_ISL_416327	28-Jan-20
G	Guangzhou, China	EPI_ISL_429080	5-Feb-20
O	France	EPI_ISL_447691	Mar-20

Among all GISAID sequences from a patient specimen from China and a collection date of December 1 to January 31, 2020, there were five cases with the D614G SNV. These five cases had 4 $\pm$ 3.3 SNVs, with a range of 1-10.

Another study of SARS-CoV-2 variants in the GISAID database was prospectively designed to identify any variant that exceeded 0.3% of all sequences at any point in time. In March 2020 the D614G variant was the first to meet this criterion and it showed domination over the D614 initial variant in each of the five continents in which it appeared and in 16 of 17 countries in which it appeared (Iceland with a well enforced lockdown and border closing being the only exception).[11]

Another study determined that, for the period of December 2019 to June 2020, the D614G mutation made the variant 31% more transmissible that the ancestral D614 strain.[12]

In August 2020, Korber et al.[13] presented evidence that there were more SARS-CoV-2 viruses circulating in the human population globally that have the G614 form of the Spike protein versus the D614 form that was originally identified from the first human cases in Wuhan, China. Follow-

[9] Isabel, S., Graña-Miraglia, L., Gutierrez, J.M. et al. Evolutionary and structural analyses of SARS-CoV-2 D614G spike protein mutation now documented worldwide. Sci Rep 10, 14031 (2020). https://doi.org/10.1038/s41598-020-70827-z

[10] https://nextstrain.org/ncov/gisaid/global?l=clock

[11] https://www.cell.com/cell/pdf/S0092-8674(20)30820-5.pdf

[12] Leung Kathy, Pei Yao, Leung Gabriel M, Lam Tommy TY, Wu Joseph T. Estimating the transmission advantage of the D614G mutant strain of SARS-CoV-2, December 2019 to June 2020. Euro Surveill. 2021;26(49):pii=2002005. https://doi.org/10.2807/1560-7917.ES.2021.26.49.2002005

[13] https://www.cell.com/cell/pdf/S0092-8674(20)30820-5.pdf

up studies showed that patients infected with G614 shed more viral nucleic acid compared with those with D614, and G614-bearing viruses show significantly higher infectious titers in vitro than their D614 counterparts.

A review[14] presented an overall picture of the impact of D614G mutation on virus transmission, elucidating the underlying mechanisms of D614G in virus pathogenicity.

Sixteen mammalian species have been identified that can be infected with the G614/FCS phenotype and two species that can be infected with both phenotypes

According to records in the online database GISAID, SARS-CoV-2 virus sequences with the G614/FCS phenotype have been recovered from the following animal hosts: Aonyx cinereus (otter; n=5), Canus lupus familarus (n=28), Chlorocebus sabaeus (monkey; n=1), Felis catus (n=70), Gorilla gorilla (n=3), Mesocricetus auratus (hamster; n=2), Mustela lutreola (mink; n=23), Mustela putorius furo (mink; n=2), Neovision vision (mink; Denmark; n=14), Panthera leo (lion; n=29), Panthera tigris (tiger; n-17), Panthera tigris jacksoni (n=13), Panthera tigris sondaica (n=1), Panthera uncia (n=3), Prionailurus bengalensis euptilurus (leopard; n=1), Rhinolophus affinis (bat; n=1).

In The Netherlands, there were reports of 956 minks infected with SARS-CoV-2 with 43 infections with the original D614/FCS. And of 37 infected lions, Panthera leo, eight were infected with the original D614/FCS.

Rabbits are susceptible to infection with G614/FCS CoV-2

The susceptibility of rabbits to SARS-CoV-2 was demonstrated with the G614/FCS variant, which excrete infectious virus from the nose and throat upon experimental inoculation.[15]

D614G rapidly arises in single passage in cats infected with wildtype SARS-CoV-2

In an experiment of a single passage of intranasally administered D614 virus to six cats, at 1-3 days post dosing, deep sequencing showed that two of five cats had the D614G SNV at 19% and 98%. The cat with fixation of D614G was able to transmit the same phenotype to another cat, causing a 99% D614G infection.[16]

[14] Chenxi Wang, You Zheng, Zubiao Niu, Xiaoyi Jiang, Qiang Sun, The virological impacts of SARS-CoV-2 D614G mutation, Journal of Molecular Cell Biology, Volume 13, Issue 10, October 2021, Pages 712–720, https://doi.org/10.1093/jmcb/mjab045

[15] Mykytyn AZ, Lamers MM, Okba NMA, et al. Susceptibility of rabbits to SARS-CoV-2. Eme rg Microbes Infect. 2021;10(1):1-7. doi:10.1080/22221751.2020.1868951

[16] Bashor L, wt. al. SARS-CoV-2 evolution in animals suggests mechanisms for rapid variant selection. bioRxiv [Preprint]. 2021 Mar 9:2021.03.05.434135. doi: 10.1101/2021.03.05.434135. Update in: Proc Natl Acad Sci U S A. 2021 Nov 2;118(44):e2105253118. doi: 10.1073/pnas.2105253118. PMID: 33758844; PMCID: PMC7987003.

Laboratory engineering D614G into the first US patient's sequence, WA1, enhances infectivity and transmission

A paper[17] entitled, "Spike mutation D614G alters SARS-CoV-2 fitness," sought to examine the effect of adding the D614G mutation to the WA1 virus, the first patient specimen in the US. This patient was a student in Seattle, WA who returned from Wuhan after the year end holiday break and, despite never having visited the Hunan Seafood Market nor being in contact with anyone in the market, became infected with SARS-CoV-2. His strain was Lineage A and so was ancestral (by two mutations or SNVs) from the market cases, which were all Lineage B. His strain also have the D614 ancestral sequence.

They engineered the spike D614G substitution in the USA-WA1/2020 SARS-CoV-2 strain, and found that it enhances viral replication in human lung epithelial cells and primary human airway tissues by increasing the infectivity and stability of virions. Hamsters infected with SARS-CoV-2 expressing spike(D614G) (G614 virus) produced higher infectious titers in nasal washes and the trachea, but not in the lungs, supporting clinical evidence showing that the mutation enhances viral loads in the upper respiratory tract of COVID-19 patients and may increase transmission.

Baric et. al. show that even with a 10:1 ratio of D614 to G614, the G614 virus can out compete the ancestral virus in human epithelial cell cultures after three passages

A paper[18] from Baric's lab entitled, "SARS-CoV-2 D614G variant exhibits efficient replication ex vivo and transmission in vivo," compared the replication fitness between the two variants, by conducting competition assays in primary airway epithelium (LAE) cultures by infecting simultaneously with both viruses. After three continuous passages at 72-hour intervals, the D614G variant became dominant in the cultures regardless of whether the WT virus was at a 1:1 or 10:1 ratio over the isogenic D614G mutant. These data suggest the D614G substitution enhances SARS-CoV-2 replication fitness in the primary epithelial cells, with an advantage in the upper respiratory tract epithelial cells in nasal and large (proximal) airway epithelia that express higher amounts of human ACE2 (hACE2) receptor.

The only documented mink-to-human transmission is G614 not D614: European mink farm outbreaks of D614 and G614 phenotypes establish animal-to-human transmission of the D614G phenotype but fail to demonstrate transmission of the D614 phenotype

In a groundbreaking paper on animal-to-human transmission entitled, "Transmission of SARS-CoV-2 on mink farms between humans and mink and back to humans,"[19] Oude Munnink et al. used whole-genome sequencing of outbreaks on 16 mink farms and the humans living or working

[17] Plante, J.A., Liu, Y., Liu, J. et al. Spike mutation D614G alters SARS-CoV-2 fitness. Nature 592, 116–121 (2021). https://doi.org/10.1038/s41586-020-2895-3

[18] Hou YJ, et. al., SARS-CoV-2 D614G variant exhibits efficient replication ex vivo and transmission in vivo. Science. 2020 Dec 18;370(6523):1464-1468. doi: 10.1126/science.abe8499. Epub 2020 Nov 12. PMID: 33184236; PMCID: PMC7775736

[19] Bas B. Oude Munnink et al. Transmission of SARS-CoV-2 on mink farms between humans and mink and back to humans. Science371,172-177(2021). DOI:10.1126/science.abe5901

on these farms to study the introduction of SARS-CoV-2 onto these farms and further transmission. The phenotype of the viruses on each farm was homogeneous with respect to the 614 phenotype, with twelve G614 and four D614 outbreaks. This is similar to a 94% D614 phenotype in 800 human specimens collected in The Netherlands from April 23 to June 21, 2020, the timing of the mink farm sampling, and reported to GISAID. This demonstrates that the D614G phenotype is not necessary for a mink infection from a human-to-mink transmission.

It also documents that some limited mink-to-mink transmission can occur with the D614 phenotype. Specifically, the Text-Table below shows that, on farms in which the D614 phenotype was found, there was a limited range and mean number of mutations seen. Given the mutation rate within a single animal-to-animal transmission, these numbers support, at most, two transmissions.

Mink Farm	Mutations (Range; Mean)
NB2	0-8; 3.6
NB5	NA
NB10	0-3; 1.1
NB15	0-2; 0.6

This is a similar level of short-term stability during transmission in humans, where the 95% CI for the emergence of the D614G phenotype, based on phylogeny trees, was 1.4-3.8 mutations and therefore does not inform the long-term stability of the D614 phenotype in the mink population.

With respect to mink-to-human transmission, the following Text-Table, contains the 18 documented mink-to-human transmissions, based on the observation that the virus sequences clustered with the sequences from the mink at the respective farm in generated phylogenetic trees.

Mink Farm	Cluster	Animal-to-Human Spread	614 Phenotype
NB3	A	5	G
NB12	A	5	G
NB8	A and D	4 (also worked at NB12)	G
NB13	A	3	G
NB7	C	2	G
NB1	A	1	G
NB9	C	1	G
NB14	C	1	G
NB4	A	0	G
NB6	C	0	G
NB11	E	0	G
NB16	A	0	G
NB2	B	0	D
NB5	D	0	D
NB10	D	0	D
NB15	D	0	D

As noted in the Text-Table, all 18 mink-to-human infections were the D614G/FCS phenotype. Given that 12 of the 16 farms have the G614 phenotype to begin with, the probability that all

infections would be the G614 phenotype, if transmission was simply a matter of initial phenotype, is $(3/4)^{18}$ or a p value of 0.006, a statistically significant finding.

The conclusion from this paper is that, while the human-to-mink transmission can be either phenotype at position 614, only the D614G/FCS phenotype can support mink-to-human transmission.

G614 outcompetes D614 in hACE2 mice following single intranasal administration

Compared to hACE2-expressing mice, wildtype mice had 100- to >1000-fold lower titers of SARS-CoV-2 following intranasal administration. Competition studies in which equal numbers of D614 and G614 virus particles were administered to eight mice and then viral load monitored during life and organ load at sacrifice, the G614 variant always outnumbered the D614 virus on day one after inoculation.[20]

G614 outcompetes D614 in intranasal single passage in hamsters and transmits significantly better in hamster-to-hamster contact settings

An experiment was conducted in which a 50/50 mixture of D614 and G614 virus particles was administered intranasally to six hamsters and then the viral load and variant ratio determined. As expected, by day 8 the ratio was 89% G614 and 11% D614 (SD=2%). In hamsters that were not inoculated but simply co-housed with the inoculated hamsters, all became infected. Interestingly, in these six animals the ratio was 96% G614 and 4% D614 (SD=2%). This difference was significant (P = <0.05) and thus strong, positive selection of the D614G variant is observed both intra-host and between hosts.

G614 outcompetes D614 in intranasal single passage in ferrets and transmits significantly better in ferret-to-ferret contact settings

A similar experiment was conducted with ferrets, in which a 50/50 mixture of D614 and G614 virus particles was administered, and viral load and variant were determined over time and in contact fetter was co-housed with the inoculated fetter. In five of the six inoculated ferrets, SARS-CoV-2G614 became the dominant variant. In addition, SARS-CoV-2 transmission occurred in four of the six ferret pairs. In each pair with successful transmission, SARS-CoV-2 G614 prevailed over SARS-CoV-2 D614. Notably, the inoculated ferret from pair 1 (in which SARS-CoV-2 D614 predominated the viral population) did not transmit virus to the contact, despite a high peak viral genome load of more than 10 million copies per ml. By contrast, the lack of transmission in pair 4 (in which SARS-CoV-2 G614 became the dominant variant) was connected to peak viral loads of below 500,000 genome copies per ml.

[20] Zhou, B., Thao, T.T.N., Hoffmann, D. et al. SARS-CoV-2 spike D614G change enhances replication and transmission. Nature 592, 122–127 (2021). https://doi.org/10.1038/s41586-021-03361-1

In summary, the competition experiment in ferrets revealed that SARS-CoV-2 G614 preferentially infected and replicated in five out of six inoculated ferrets, and the successful transmission events occurred only with SARS-CoV-2 G614.

In a related experiment, the transmission of G614 from ferrets inoculated intranasally to ferrets in a direct, co-housing cage versus a cage 10 cm distant, where only air-borne virus could be transmitted, was conducted.[21] In these experiments four of four ferrets in direct co-housing became infected while three of four ferrets were infected by air-borne particles.

In another related experiment, the transmission of SARS-CoV-2 among ferrets that were co-housed (direct contact) or via indirect contact, using adjacent cages via air-borne particles (six ferrets intranasal administration, six ferrets direct contact, and six ferrets indirect contact).[22] Here, unlike with the above experiments, while all six ferrets in direct contact became infected, none of the six ferrets with indirect contact became clinically infected or seroconverted. The authors conclude that: "(t)hese data indicate that the efficient establishment of COVID-19 clinical features in ferrets exposed to infected animals requires direct contact, recapitulating human-to-human transmission."

Unfortunately, the paper only states that the virus was "a strain that was isolated from a COVID-19-confirmed patient in South Korea in February of 2020" and does not provide the D614G phenotype of the virus. However, an examination of GISAID for all virus sequences deposited between December 1, 2019, and February 28, 2020 from South Korea identified 139 SARS-CoV-2 isolates, none of which had the D614G phenotype. It is thus very reasonable to conclude that these experiments were conducted with the D614 phenotype, providing additional support for the limited transmissibility of this phenotype in the ferret.

D614G enhances passage in human lung epithelial cell line Calu-3 by three-fold

This paper[23] engineered the spike D614G substitution in the USA-WA1/2020 SARS-CoV-2 strain and found that it enhances viral replication in human lung epithelial cells and primary human airway tissues by increasing the infectivity and stability of virions. Hamsters infected with SARS-CoV-2 expressing spike D614G virus produced higher infectious titers in nasal washes and the trachea, but not in the lungs, supporting clinical evidence showing that the mutation enhances viral loads in the upper respiratory tract of COVID-19 patients and may increase transmission.

[21] Richard, M., Kok, A., de Meulder, D. et al. SARS-CoV-2 is transmitted via contact and via the air between ferrets. Nat Commun 11, 3496 (2020). https://doi.org/10.1038/s41467-020-17367-2

[22] Kim, Young-Il et al. Infection and Rapid Transmission of SARS-CoV-2 in Ferrets. Cell Host & Microbe, Volume 27, Issue 5, 704 - 709.e2 Article Hyperlink

[23] Plante, J.A., Liu, Y., Liu, J. et al. Spike mutation D614G alters SARS-CoV-2 fitness. Nature 592, 116–121 (2021). https://doi.org/10.1038/s41586-020-2895-3

Growth of SARS-CoV-2 in TMPRSS2-deficient VERO cells leads to rapid delete of the FCS, even during the first passage

In a study of SARS-CoV-2 growth in VERO cells, within five passages there were frequent in-frame deletions of the FCS, including NSPRRAR/SVA and RAR/SVAS.[24] In fact, the FCS deletion mutants were found even in the first passage and were >50% of the virus in the second passage. In a VERO-TMPRSS2 expressing cell line, these deletions were not seen. In a related study during first passage growth in VERO-E6 cells, a 24 nt in-frame deletion was detected in over half of the subgenomic mRNAs encoding the Spike Protein and was predicted to remove the same peptide, NSPRRAR/SV, as seen in the above study.[25]

In confirmatory studies, a SARS-CoV-2 mutant lacking the furin cleavage site (ΔPRRA) in the spike protein was generated. This mutant virus replicated with faster kinetics and improved fitness in Vero E6 cells. The mutant virus also had reduced spike protein processing as compared to wild-type SARS-CoV-2. In contrast, the ΔPRRA had reduced replication in Calu3 cells and had attenuated disease in a hamster pathogenesis model.[26]

Growth of SARS-CoV-2 in human-derived cell lines or human airway organoids does not lead to FCS deletion and growth of FCS deletion variants in these cell lines is not supported

On the other hand, although the human lung epithelial Calu-3 and colon epithelial Caco-2 cell lines are known to be susceptible to SARS-CoV-2 infection, compared to WT virus, few to no Calu-3 or Caco-2 cells were susceptible to infection with any of the FCS deletion viruses.[15] It was reported that the SARS-CoV-2 furin site increases infectivity on human airway organoids (hAOs). Compared with SARS-CoV, SARS-CoV-2 more frequently, formed syncytia in hAOs.[27]

The furin cleavage site in the SARS-CoV-2 spike protein is required for transmission in ferrets[28]

Using lentiviral pseudotypes and a cell-culture-adapted SARS-CoV-2 virus with an S1/S2 deletion, the authors show that the polybasic insertion endows SARS-CoV-2 with a selective advantage in lung cells and primary human airway epithelial cells, but impairs replication in Vero

[24] Sasaki M, et' al. SARS-CoV-2 variants with mutations at the S1/S2 cleavage site are generated in vitro during propagation in TMPRSS2-deficient cells. PLoS Pathog. 2021 Jan 21;17(1):e1009233. doi: 10.1371/journal.ppat.1009233. PMID: 33476327; PMCID: PMC7853460.
[25] Davidson, A.D., Williamson, M.K., Lewis, S. et al. Characterisation of the transcriptome and proteome of SARS-CoV-2 reveals a cell passage induced in-frame deletion of the furin-like cleavage site from the spike glycoprotein. Genome Med 12, 68 (2020). https://doi.org/10.1186/s13073-020-00763-0
[26] Johnson BA, et. al. Furin Cleavage Site Is Key to SARS-CoV-2 Pathogenesis. bioRxiv [Preprint]. 2020 Aug 26:2020.08.26.268854. doi: 10.1101/2020.08.26.268854. Update in: Nature. 2021 Mar;591(7849):293-299. doi: 10.1038/s41586-021-03237-4. PMID: 32869021; PMCID: PMC7457603.
[27] Mykytyn, AZ, et. al. (2021) SARS-CoV-2 entry into human airway organoids is serine protease-mediated and facilitated by the multibasic cleavage site eLife 10:e64508 https://doi.org/10.7554/eLife.64508
[28] Peacock, T.P., Goldhill, D.H., Zhou, J. *et al.* The furin cleavage site in the SARS-CoV-2 spike protein is required for transmission in ferrets. *Nat Microbiol* **6,** 899–909 (2021). https://doi.org/10.1038/s41564-021-00908-w

E6, a cell line used for passaging SARS-CoV-2. Using engineered spike variants and live virus competition assays and by measuring growth kinetics, they found that the selective advantage in lung and primary human airway epithelial cells depends on the expression of the cell surface protease TMPRSS2, which enables endosome-independent virus entry by a route that avoids antiviral IFITM proteins. SARS-CoV-2 virus lacking the S1/S2 furin cleavage site was shed to lower titres from infected ferrets and was not transmitted to co-housed sentinel animals, unlike wild-type virus.

SARS-CoV-2 D614 transmits poorly in dogs, pigs, chickens, and ducks

In this study using D614 SARS-CoV-2 viruses, the authors investigated the susceptibility of ferrets and animals in close contact with humans to SARS-CoV-2.[29] They found that SARS-CoV-2 replicates poorly in dogs, pigs, chickens, and ducks, but ferrets and cats are permissive to infection. Additionally, cats are susceptible to airborne transmission.

SARS-CoV-2 infects raccoon dogs poorly due to their non-optimal ACE2 receptor

This study[30] was conducted by George Gao, former head of the Chinese CDC.

While the raccoon dog has been proposed as a potential host of SARS-CoV-2, there is no evidence support such a notion. In this study, they investigated the binding affinities of raccoon dog ACE2 (rdACE2) to the spike (S) protein receptor binding domain (RBD) of SARS-CoV-2 prototype (PT) and its variants. It revealed that the binding affinities of RBD from SARS-CoV-2 variants were generally lower than that of the PT RBD. Through structural and functional analyses, they found amino acids H34 and M82 play pivotal roles in maintaining the binding affinity of ACE2 to different SARS-CoV-2 sub-variants. These results suggest that raccoon dogs exhibit lower susceptibility to SARS-CoV-2 compared to those animal species with a high prevalence of SARS-CoV-2 transmission.

Raccoon dogs transmit D614G SARS-CoV-2 poorly

There is not a single raccoon dog SARS-CoV-2 infection in the GISAID database, despite the claim that there are 11 million animals being breed in China on fur farms.[31] In this study, the D614G phenotype virus was used to infect nine raccoon dogs with artificially large intranasally doses. Despite the large dose, only 66% were transiently infected. The contact animals not inoculated had an infection rate of 2 out of 3 or 66% as well. Surprisingly, none of the internal organs at autopsy showed evidence of infection nor could virus be isolated. The animals, at most, develop a very short-lived infection of the nasal cavity with minimal shedding

[29] Shi J, et. al. Susceptibility of ferrets, cats, dogs, and other domesticated animals to SARS-coronavirus 2. Science. 2020 May 29;368(6494):1016-1020. doi: 10.1126/science.abb7015. Epub 2020 Apr 8. PMID: 32269068; PMCID: PMC7164390.

[30] Luo C, Li L, Gu Y, Zhang H, Xu Z, Sun J, et al. (2024) Receptor binding and structural basis of raccoon dog ACE2 binding to SARS-CoV-2 prototype and its variants. PLoS Pathog 20(12): e1012713. https://doi.org/10.1371/journal.ppat.1012713

[31] Freuling CM, Breithaupt A, Müller T, Sehl J, Balkema-Buschmann A, Rissmann M, et al. Susceptibility of Raccoon Dogs for Experimental SARS-CoV-2 Infection. Emerg Infect Dis. 2020;26(12):2982-2985. https://doi.org/10.3201/eid2612.203733

Cynomolgus macaques can be infected with SARS-CoV-2 D614 that has an additional mutation at S686G[32]

To compare its pathogenesis with that of previously emerging coronaviruses, the authors inoculated cynomolgus macaques with SARS-CoV-2 or Middle East respiratory syndrome (MERS)–CoV and compared the pathology and virology with historical reports of SARS-CoV infections. In SARS-CoV-2–infected macaques, virus was excreted from nose and throat in the absence of clinical signs and detected in type I and II pneumocytes in foci of diffuse alveolar damage and in ciliated epithelial cells of nasal, bronchial, and bronchiolar mucosae. In SARS-CoV infection, lung lesions were typically more severe, whereas they were milder in MERS-CoV infection, where virus was detected mainly in type II pneumocytes. These data show that SARS-CoV-2 causes COVID-19–like disease in macaques and provides a new model to test preventive and therapeutic strategies.

Bat-to-bat transmission in fruit bats (*Rousettus aegyptiacus*) of G614 SARS-CoV-2 is significantly lower than for ferrets and would be unlikely to be a reservoir host for the D614/FCS phenotype

As noted above, while the D614 variant can infect ferrets it does not transmit to contact ferrets. In these experiments fruit bats and ferrets were infected with the G614 phenotype and both infection and transmission to contact animals assessed.[33] Only seven of nine inoculated bats became infected and none of the three contact bats were found to shed virus, although one of three was minimally infected.

On the other hand, all contact ferrets became highly infected, and viruses were found to be shed in high quantities. Given the previous finding that the D614/FCS phenotype could not be transmitted in ferrets, this finding that fruit bats are poorer at transmitting the G614 variant makes it unlikely that the fruit bat could be a reservoir host for the less transmissible D614/FCS phenotype.

The G614 phenotype outperforms the D614 phenotype in ACE2-humanized laboratory mice[34]

Another Baric lab study on the D614G adaptation[35] was conducted in which they engineered a SARS-CoV-2 variant contained this substitution. The variant exhibited more efficient infection, replication, and competitive fitness in primary human airway epithelial cells but maintained similar morphology and in vitro neutralization properties, compared with the ancestral wild-type virus. Infection of human angiotensin-converting enzyme 2 (ACE2) transgenic mice and Syrian hamsters

[32] Rockx B, et. al. Comparative pathogenesis of COVID-19, MERS, and SARS in a nonhuman primate model. Science. 2020 May 29;368(6494):1012-1015. doi: 10.1126/science.abb7314. Epub 2020 Apr 17. PMID: 32303590; PMCID: PMC7164679.
[33] Schlottau K, Rissmann M, Graaf A, et al. SARS-CoV-2 in fruit bats, ferrets, pigs, and chickens: an experimental transmission study. Lancet Microbe. 2020;1(5):e218-e225. doi: 10.1016/S2666-5247(20)30089-6
[34] https://science.sciencemag.org/content/sci/370/6523/1464.full.pdf
[35] Hou, YJ, et. al. SARS-CoV-2 D614G variant exhibits efficient replication ex vivo and transmission in vivo. Science 370,1464–1468 (2020).

with both viruses resulted in similar viral titers in respiratory tissues and pulmonary disease. However, the D614G variant transmitted significantly faster and displayed increased competitive fitness than the wild-type virus in hamsters. These data show that the D614G substitution enhances SARS-CoV-2 infectivity, competitive fitness, and transmission in primary human cells and animal models.

Sixteen of eighteen animal species known to be present in the Hunan Seafood Market in 2019 have no SARS-CoV-2 infections reported anywhere in the world. One species has 68 D614G infections and no D614G infections. The other single species with infections are the minks infected on farms in The Netherlands (*supra*)

Xiao et al.[36] studied wild animal sales across four markets in Wuhan (including the Huanan market) from May 2017 to November 2019. Average monthly sales of different species across the four markets are shown below:

Species on sale	Monthly mean (and SD) number of individuals sold	Price (mean ± SD) $ per individual
Mammals		
Raccoon dog (*Nyctereutes procyonoides*)[W,R,F,†]	38.33 ± 17.24 (n = 30)	63.32 ± 15.46 (n = 5)
Amur hedgehog (*Erinaceus amurensis*)[R,F,†]	332.14 ± 190.62 (n = 28)	2.66 ± 0.41 (n = 5)
Siberian weasel (*Mustela sibirica*)[W,R,F,†]	(10.06 ± 12.09, n = 31)	11.24 ± 3.07 (n = 5)
Hog badger (*Arctonyx albogularis*)[W,R,F,†]	(6.81 ± 5.37, n = 31)	72.79 ± 34.08 (n = 5)
Asian badger (*Meles leucurus*)[W,R,F,†]	12.24 ± 7.39 (n = 29)	59.77 ± 15.89 (n = 5)
Chinese hare (*Lepus sinensis*)[W,R,F,†]	168.96 ± 89.06 (n = 29)	16.87 ± 2.88 (n = 5)
Pallas's squirrel (*Callosciurus erythraeus*)[R,P,†]	16.52 ± 4.87 (n = 23)	25.74 ± 7.59 (n = 5)
Masked palm civet (*Paguma larvata*)[F,†]	10.69 ± 8.42 (n = 29)	62.73 ± 15.25 (n = 5)
Chinese bamboo rat (*Rhizomys sinensis*)[F,†]	42.76 ± 20.68 (n = 29)	18.64 ± 7.58 (n = 5)
Malayan porcupine (*Hystrix brachyura*)[F,†]	10.00 ± 0.00 (n = 29)	68.06 ± 14.23 (n = 5)
Chinese muntjac (*Muntiacus reevesi*)[F,†]	10.00 ± 0.00 (n = 29)	142.62 ± 49.67 (n = 5)
Coypu (*Myocastor coypus*)[F]	5.00 ± 0.00 (n = 29)	28.70 ± 5.08 (n = 5)
Marmot (*Marmota himalayana*)[F]	15.00 ± 4.29 (n = 20)	81.37 ± 11.70 (n = 5)
Red fox (*Vulpes vulpes*)[F,†]	30.00 ± 0.00 (n = 25)	60.96 ± 21.68 (n = 5)
Mink (*Neovison vison*)[F]	10.37 ± 1.92 (n = 27)	34.62 ± 14.78 (n = 5)
Red squirrel (*Sciurus vulgaris*)[R,P,†]	16.43 ± 9.51 (n = 28)	26.04 ± 8.14 (n = 5)
Wild boar (*Sus scrofa*)[W,R,F,*,†]	(4.17 ± 5.77, n = 29)	319.57 ± 55.95 (n = 5)
Complex-toothed Flying Squirrel (*Trogopterus xanthipes*)[F,P,†]	5.17 ± 27.85 (n = 29)	28.11 ± 9.64 (n = 5)

The GISAID database was interrogated for evidence of infections in these species to determine:

1. Are these species able to be infected in the wild;
2. If so, are the infections the ancestral D614 or are the epidemic D614G virus.

If a species was found that had numerous D614 infections it would become a good candidate for an intermediate host for the pre-human SARS-CoV-2 virus. The results of this analysis is shown in this Text-Table:

[36] Xiao, X., Newman, C., Buesching, C.D. et al. Animal sales from Wuhan wet markets immediately prior to the COVID-19 pandemic. Sci Rep 11, 11898 (2021). https://doi.org/10.1038/s41598-021-91470-2

Animal Species	GISAID Sequences (D614/Total); Location
Raccoon dog	None
Amur Hedgehog	None
Siberian Weasel	0/68; Spain
Hog Badger	None
Asian Badger	None
Chinese Hare	None
Pallas's Squirrel	None
Masked Palm Civet	None
Chinese Bamboo Rat	None
Malayan Porcupine	None
Chinese Muntjac	None
Coypu	None
Marmot	None
Red Fox	None
Mink	42/1376 or 3%; Neatherlands
Red Squirrel	None
Wild Boar	None
Flying Squirrel	None

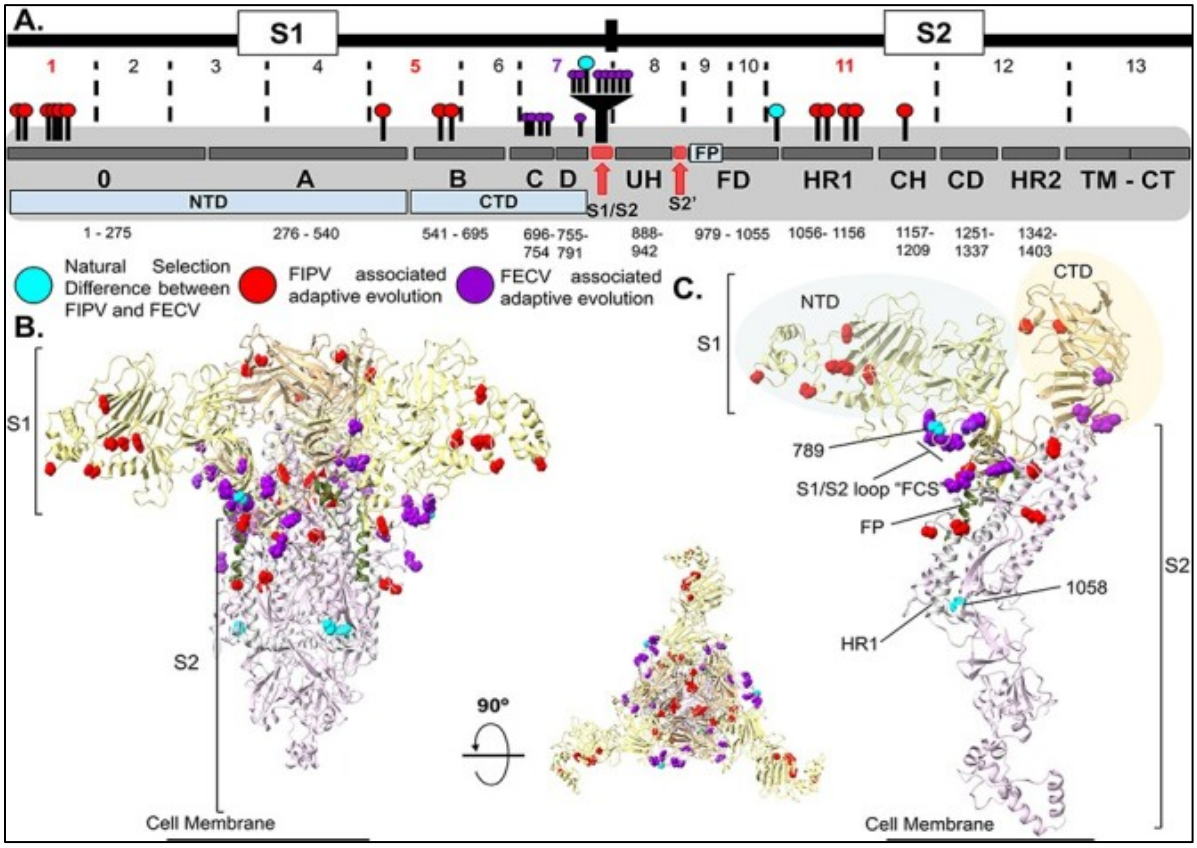
As shown above, 16 of the 18 animal species found in the Wuhan market have no viral sequences in the GISAID database. While not definitive, this absence of any infected animals suggests that these species are not naturally infected with a SARS2-related virus. The database does contain a report from Spain of 68 Siberian Weasels infected with SARS-CoV-2. All of these were the D614G infection and were probably a reverse human-host zoonosis. The mink data from The Netherlands has been discussed above. Although 3% of the infections were the ancestral D614 virus, tellingly, this virus was not transmitted within the farms. There simply is no evidence of the D614 virus being in a stable animal reservoir. This requires the alternative hypothesis to be accepted, until evidence to the contrary is produced, that the D614 virus much have come from a laboratory.

An experiment of nature confirms the need for stabilization of the S1 in viruses with an FCS: The feline coronavirus exists in two forms, one having an FCS and one without an FCS, and there is selection pressure within the adjacent S1 for amino acid substitutions in the FCS variant

Feline coronaviruses (FCoVs) commonly cause mild enteric infections in felines worldwide (termed feline enteric coronavirus [FECV]), with around 12 per cent developing into deadly feline infectious peritonitis (FIP; feline infectious peritonitis virus [FIPV]). A paper from 2023[37]

[37] Zehr JD, et. al., Natural selection differences detected in key protein domains between non-pathogenic and pathogenic feline coronavirus phenotypes. Virus Evol. 2023 Mar 15;9(1):vead019. doi: 10.1093/ve/vead019. PMID: 37038392; PMCID: PMC10082545.

used state-of-the-art molecular evolutionary genetic statistical techniques to identify and compare differences in natural selection pressure between FECV and FIPV sequences, as well as to identify FIPV- and FECV-specific signals of positive selection. They identified two sites exhibiting differences in natural selection pressure between FECV and FIPV: one within the S1/S2 furin cleavage site (FCS) and the other within the fusion domain of Spike. They also found fifteen sites subject to positive selection associated with FIPV within Spike, eleven of which have not previously been suggested as possibly relevant to FIP development. These sites fall within Spike protein subdomains that participate in host cell receptor interaction, immune evasion, tropism shifts, host cellular entry, and viral escape. There were fourteen sites (twelve novel sites) within Spike under positive selection associated with the FECV phenotype, almost exclusively within the S1/S2 FCS and adjacent to C domain.



The finding of mutations in the N-terminal portion of the spike protein from the S1/S2 junctional FCS is similar to the D614G highly selected mutation in SARS-CoV-2 and is consistent with the hypothesis that the destabilization of the spike protein S1 following cleavage at the S1/S2 junction is compensated for with adjacent mutations which stabilize the S1 following cleavage, preventing its dissociation from the virus.[38]

[38] @Martinasisters from the social media site X suggested this analysis be conducted.

By 2021, other stabilizing mutations provided the opportunity for the D614G to revert to the native salt bridge structure present before the FCS was added

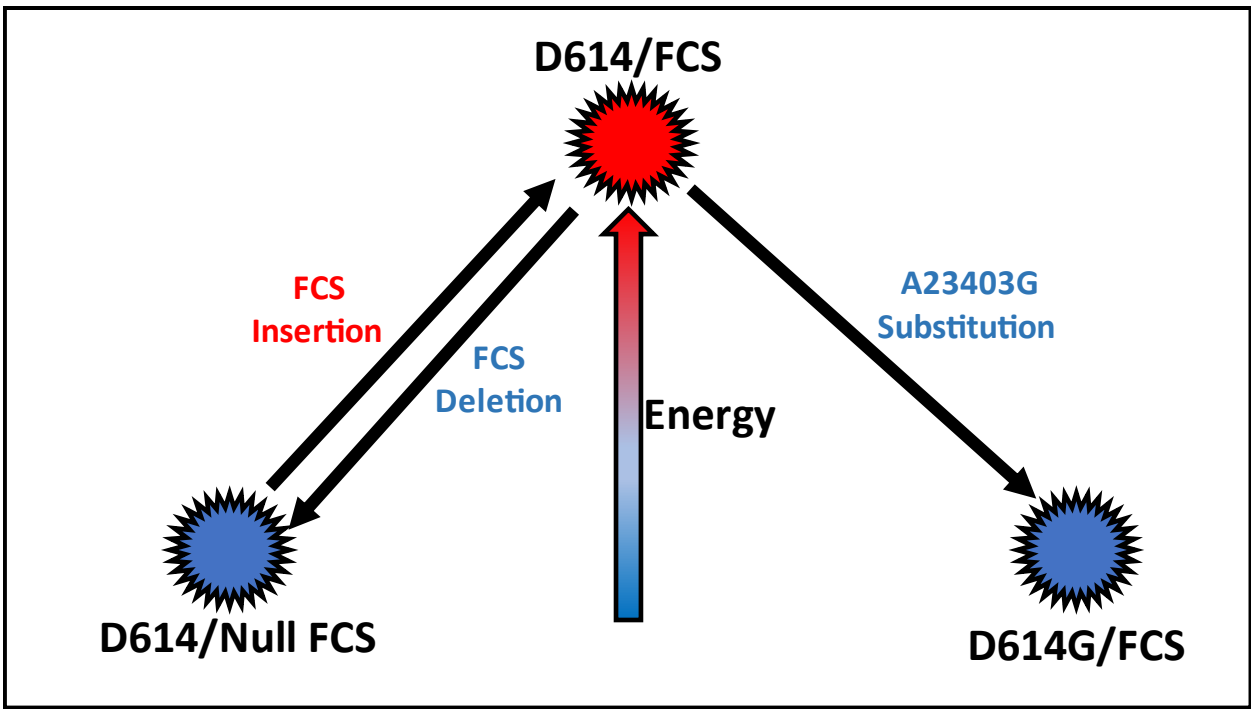
By March 2021, the ancestral D614 was very rarely sampled. D614G confers a fitness advantage in terms of transmissibility, and global samples had almost entirely shifted to the mutated form by early summer of 2020. Still, the D614G mutation may come at a cost for the virus, as some have found the ancestral D614 form to be more resistant to neutralization by sera. A 4- to 6-fold increase in vaccine sera sensitivity was observed for D614G in one study, while in another, an average 1.7-fold difference was observed among sera from hamsters infected with D614 virus against the D614G variant. However, not all studies find such a difference. For example, Hou and colleagues did not see a significant difference between the two forms in terms of neutralization sensitivity to human convalescent sera. As the virus is increasingly confronted with convalescent and vaccine sera over the course of 2021, the greater neutralization sensitivity of the D614G form (if this is indeed the case) may come to outweigh its increased transmissibility as a selective force at the population level, and D614 may begin to re-emerge. Of note in this regard, the ancestral D614 is part of the Spike signature of the VOI in the A23.1 lineage that recently emerged from Uganda. D614 has also recently resurfaced in combination with Δ69/70 and with Δ144. A small number of interesting D614 sequences sampled in Wales and England carry both Δ69/70 and Δ144 as well as a third distinctive 2-amino-acid deletion, Δ243–244.

These observations do not negate the importance of D614G at the beginning of the pandemic.

DISCUSSION

Model of the metastable nature of the D614/FCS phenotype.

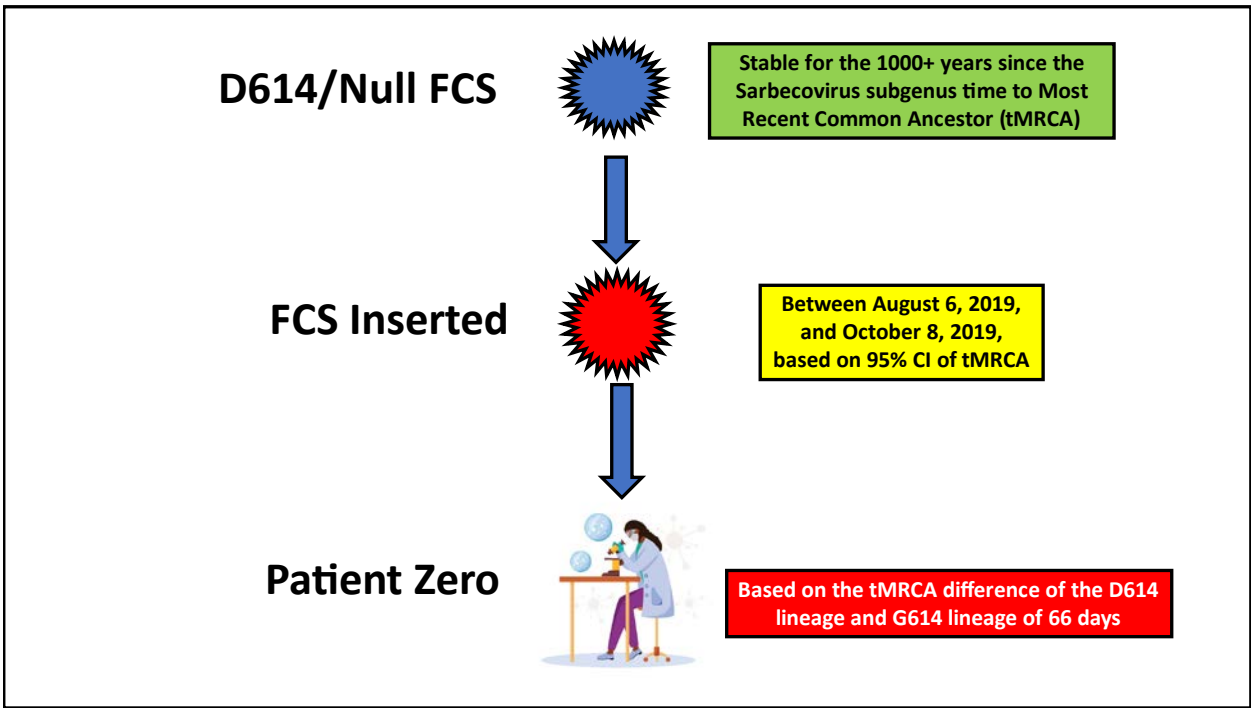
Figure 1 is a model of the metastable nature of the SARS-CoV-2 D614/FCS Phenotype.



Here I have shown that the D614/Null FCS is the ancestral phenotype and was therefore the progenitor SARS-related coronavirus to which the FCS was inserted. I have also shown that the resulting D614/FCS virus is metastable and that either the D614G substitution or the FCS deletion happens quickly. In the lab, and especially VERO cells which lack furin, the FCS deletion is the preferred event.

The time between FCS insertion and laboratory 'Patient Zero' is short, between 36 and 99 days

Given that the difference in tMRCA between the D614 and D614G viruses for human infections is 66 days, with a 95% CI of 36 to 99 days, one could opine that the time between the insertion of the FCS and the first human infection is about that time frame, if the timing of the conversion in the laboratory is the same for patient transmission. While this is not a strong argument, it does provide a very clear target for the timing of the laboratory experiments that led to the laboratory-acquired infection. This figure illustrates this point:



Human-to-human transmission drives conversion of D614

The D614 amino acid is present in a highly conserved 11 amino acid motif, "vavlyqdvnct" which is present in an un-substituted state in all the sarbecovirus strains of coronavirus annotated prior to the pandemic except in the ACQ82725 strain where it occurs as "vavlyqgvnct". These coronavirus strains containing "vavlyqdvnct" include 68 human SARS-CoV (known as SARS at that time) and several bat, and civet SARS-like strains. Recently reported RaTG13 bat and Pangolin strains and the original Wuhan strain spike proteins also had "vavlyqdvnct" in an un-substituted form. The motif is densely hydrophobic relative to the 11-amino acid stretches on either side of the motif (1). The D to G substitution further increased the hydrophobicity of the motif by approximately 38%. The stable longevity of the motif establishes that this was the form present when the FCS was first introduced into what would become SARS-CoV-2.

The FCS-cleaved Spike Protein of a D614 virus leads to S1 shedding (perhaps assisted by antibody binding), removing the RBD, and rendering the virus inert. This is a instability property of the virus and has nothing to do with the host species. This precludes finding a host species that can effectively propagate and transmit the D614/FCS.

To compensate for this, the D614G substitution rapidly and independently evolved because it re-establishes S1 stability, restoring the RBD to the viruses and rendering them infective.

In laboratory cell culture, the inefficiency of a D614/FCS virus can still be propagated by using large inoculums. Since Spike-directed antibodies might further destabilize the S1 that has been furin-cleaved, this might be an explanation for the ability to maintain the D614 phenotype in cell culture.

CONCLUSION

The inherent instability of the D614/FCS and its rapid conversion to D614G/FCS under all conditions of transmission outside of a laboratory, establishes that SARS-CoV-2 could not have been a zoonotic spillover and that the time from when the FCS was inserted into the SARS-CoV-2 backbone in a lab and the first laboratory-acquired infection is short, within weeks or, at most, a few months. This timing permits a focused investigation into collateral events in the second half of 2019 in and around Wuhan, China.