 doi:10.20944/preprints202207.0100.v1

Review

From minor mutant to dominance, the evolution of SARS-CoV-2 Variants

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Abstract: The successive waves of the Covid-19 pandemic are driven by SARS-CoV-2 variants that reached critical detection levels in different parts of the world. But how evolved the Wuhan virus since its detection in December 2019 into the Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529) variants of concern? This is a story of mice and men, of up to 1,000,000 infected cells in one person, where each cell produces between 10^5 and 10^6 viral RNAs, of immune-compromised patients, the digestive tract and viral recombination.

Keywords: omicron, alpha, delta, deltacron, recombination, RNA editing, intra-host variants, tropisms, oxidative damage, Wuhan

1. Introduction

In January 2020, a 20 yr old female takes a train ride from Wuhan to Kunking in Yunnan Province, China before returning home to Trichur in the South Indian state of Kerala [1], but unknowingly the SARS-CoV-2 virus multiplies in her cells. Novel mutations arise in the viral single-stranded (+) RNA genome through (i) replication errors, (ii) cellular RNA editing enzymes, (iii) oxidative stress and (iv) recombination, while up to one million viral particles (10^5-10^6 virons/infected cell) accumulate in each of her 10,000 to 1,000,000 infected cells [2] (Figure 1). In the process, minor mutations crop up which divert from the original genome. The intracellular mutant population around this master sequence is the quasispecies or mutant cloud of the virus, a term coined by Manfred Eigen in 1971 [3] [4] [5]. Although the term quasispecies is typically used to describe the total virus diversity within one person, the mutant generating mechanisms occur intracellularly. Each person is therefore a mosaic of between 10^4 and 10^6 infected cells with distinct genome clouds. These intra-host mutations are known as single nucleotide variations (iSNVs). While the vast majority of iSNVs are silent or cleared due to their deleterious effects, a few will prevail as they promote viral fitness. The positively selected iSNVs spread first slowly in local communities before traveling across countries or continents. Once they are repeatedly detected in patient samples they are referred to as "fixed" and will be recorded at population level as novel single nucleotide polymorphisms (SNPs). A group of genetically closely related genomes that derived from a common ancestor by acquiring novel SNPs is a lineage. A variant can be a lineage or a closely related group of lineages converging from more than one ancestral genome. A lineage or variant differs from the original Wuhan strain by a set of hallmark mutations. Depending on the epidemiological and clinical impact of the SNPs, public health organizations designate them as a Variant Being Monitored (VBM), a Variant of Interest (VOI) or a Variant of Concern (VOC). As of May, 20th 2022, 10,937,289 SARS-CoV-2 sequences were submitted to the GISAID database [6].

Although we talk about variants as if they were defined entities, they are in fact closely related and continuously evolving genome populations with different patterns of minor mutations around a core, variant-defining sequence. For example, the first person diagnosed with the Wuhan strain in Paris was repeatedly tested between January, 29th

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2020 and February, 10th 2020. Each sample contained a median of 38 minor mutations with a frequency of ≥1% which varied between lower (sputum) and upper respiratory tract (nasopharyngeal swaps). In total 233 intra-host single nucleotide variations (iSNVs) were recorded that randomly scattered along the viral genome [7].

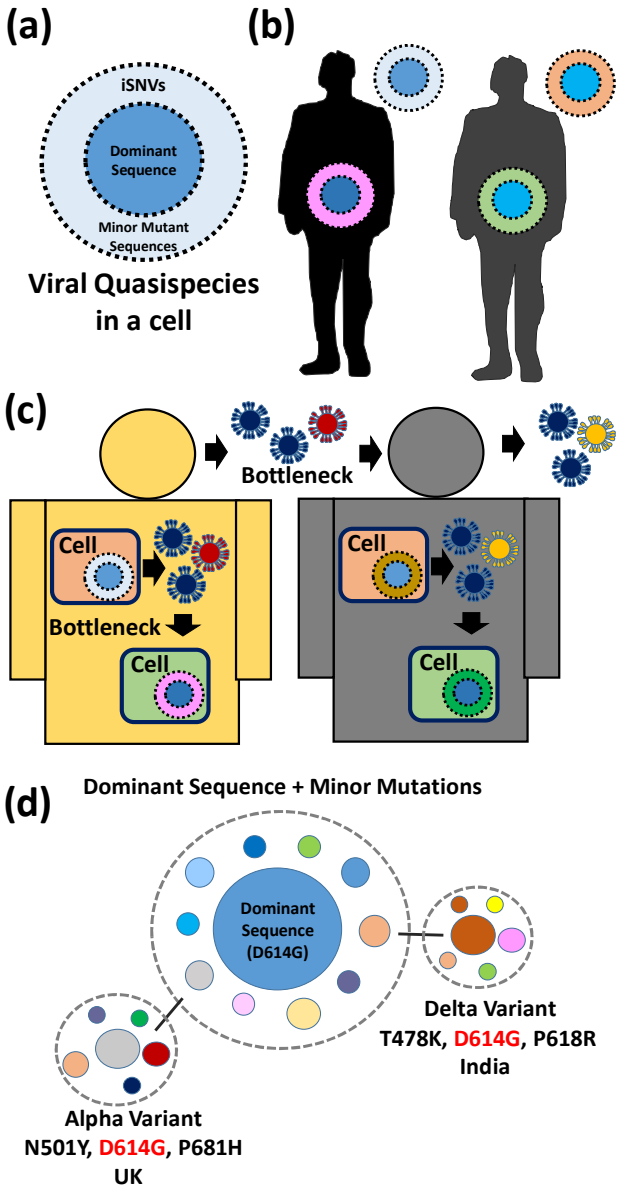

Figure 1: The genome cloud (quasispecies) nature of the SARS-CoV-2 virus. (a) Replication errors, genetic exchange and cellular editing generate a population of viral genomes in an infected cell that is dominated by the lineage-defining sequence with minor mutations known as intra-host single nucleotide variations (iSNVs). This intracellular genome population is a genome cloud or quasispecies, although the term quasispecies is also used to describe the total sequence diversity within one host. (b) Given the different selection pressures, the composition of iSNVs is unique to infected cells and in total to different hosts. (c) The bottleneck is the dramatic reduction in sequence diversity when only a small number of viral genomes are passed on to either a new cell (in-

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tra-host bottlenecks) or a new host (intra-host bottleneck). Small bottlenecks (i.e. a small number of transferring viruses) tend to select rarer genomes. (d) Novel variants emerge at different times and at different locations as illustrated for the fitness-enhancing mutation aspartate-614 to glycine (D614G). The D614G mutation in the viral Spike receptor appeared independently in at least two different countries (Germany and China) forming rapidly the dominant sequence species from which again in different countries novel variants emerged as illustrated for the Alpha and Delta variants. The D614G mutation promotes the open conformation of each subunit in the trimeric Spike receptor therefore promoting its binding to the human Ace2 protein at the surface of our cells [7]

iSNVs are detected in up to 95% of Covid-19 cases (range 68% to 95%). The mean is 8 iSNVs per patient ranging from one or two to more than twenty [8] [9]. That only such a modest number is found although a single patient may host up to 10^{12} viral genomes is down to the dramatic reduction in sequence diversity when new cells are infected. Only a few viruses with high fitness transfer their genomes to a new host cells as they promote viral fitness or allow the virus to escape neutralization by the immune system. The vast majority of the quasispecies genomes is however lost. This reduction in sequence diversity is known as the bottleneck. Bottlenecks exist within patients as well as between hosts. The number of SARS-CoV-2 virons transmitted between people is probable less than 10 [10] [11] (Figure 1). Only iSNVs which travers successfully through these bottlenecks due to fitness gains become eventually fixed as SNPs. The estimated evolution rate of SARS-CoV-2 is ~3 novel SNPs per month (6,50 x 10^{-4} substitutions/site/year) [12] [13]. This agrees well with the average number of SNPs in the Alpha (29,7), Gamma (29,1), Beta (28,4) and Delta (35,4) variants that emerged ~10 months (alpha), ~11 months (beta, delta) and ~12 months (gamma) after the first detection of the Wuhan strain in December 2019 [14]. The clear exception is intriguingly the omicron variant with its average number of 53,3 mutations.

2. The singe-stranded RNA Genome of SARS-CoV-2

The genome of SARS-CoV-2 is a single-stranded 5´to 3´ (+) sense RNA of 29903 nt with a 5` CAP structure (^{m7}GpppNm-RNA; 7-methylguanosine linked to the 5´-nucleosid with a methyl group on ribose 2´-O (Nm)) and a 3´-polyA tail that acts as a messenger RNA (mRNA) once the virus has entered a human cell (NCBI Reference Sequence: NC_045512.2) [15][16] (Figure 2) . Its 5´-untranslated region (UTR) (1-265nt) is followed by two long open reading frames, ORF1a and ORF1b (266-21555nt), separated by a −1 ribosome frameshift immediately upstream of the ORF1a stop codon. Both polypetides are the first to be translated. The viral 3C-like proteases NSP3 and NSP5 cleave both the ORF1a polypeptide into 11 Non-Structural Proteins (NSP1-11) and the ORF1b polypeptide into 5 proteins (NSP12-16) [17]. In addition to the full-length genomic RNA, nine major sub-genomic mRNAs are produced. They encode (i) the viral Spike receptor (gene S (ORF 2); 21563-25384nt) that binds to human Ace2 [18], (ii) the ion channel ORF3a (25393-26220nt) required for the release of viral particles from infected cells [19], (iii) the envelope protein E (ORF4; 26245-26472nt) that resides in the viral membrane and interferes with cell polarity and cell-cell junction integrity [20], (iv) the glycosylated membrane protein M (ORF5; 26523-27191nt) important for the assembly of new viral particles [21], (v) ORF6 (27202-27387nt) involved in antagonizing interferon signaling [22], (vi) ORF7a (27394-27759nt), (vii) ORF7b (27756-27887nt), (viii) ORF8 (27894-28259nt) required for immune evasion [23], and (viii) the nucleocapsid protein N (28274-29533nt) needed to pack the RNA into the particles [24]. The N gene contains with ORF9b and ORF9c two cryptic genes [25] [26]. Between the 3´-UTR (29675-29903nt) and the N gene resides ORF10 (29558-29674nt) with unknown function [27] (Figure 2).

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Within 8 h post-infection, the viral RNAs comprise almost 80% of the total messenger RNAs and 34% of the mRNA fragments bound by ribosomes in the cell [28]. The viral protein NSP1 binds the 5′-UTR thereby protecting the viral RNA from degradation that is the fate of the majority of host mRNAs upon infection [28]. It also attaches itself to the 40S subunit of host ribosomes to ensure preferred translation of the viral mRNAs [29]. Viral replication takes place within a reticulovesicular network at the endoplasmic reticulum induced by NSP4 and NSP6 [30] [31] [32]. The hexameric NSP7-NSP8 ring provides the RNA primer for the Replication-Transcription Complex (RTC) to synthesize the 3′-5′antisense RNA, the template for the production of the viral genome and the sub-genomic mRNAs [33] [17]. The RTC forms around NSP12, the catalytic subunit of the RNA-dependent-RNA-Polymerase (RdRP) upon binding of one NSP7 protein and two isoforms of NSP8 [34]. Within RTC, Nsp14 provides the 3′-5′exoribonuclease proofreading activity [35], Nsp13 the helicase function [36], Nsp9 mediates RNA binding, Nsp15 acts as uridylate-specific endoribonuclease and Nsp16 as 2′-O-ribose-methyltransferase [17]. NSP10, NSP13, NSP14 and NSP16 collaborate to generate the 5′-cap structure [17]. Protein coding regions account for 97,85% (29261/29903) of the genome.

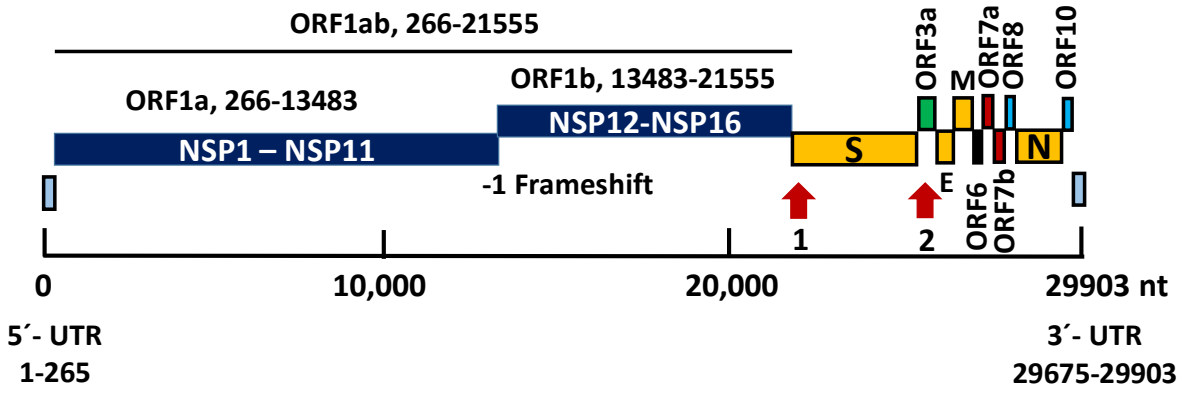

Figure 2: The single-stranded RNA genome of SARS-CoV2. The gene organization along the 29903nt long genome is shown. The four structural proteins Spike (S), Envelope (E), Membrane (M) and Nucleocapsid (N) are marked in yellow. The -1 frameshift that separates ORF1a and ORF1b is indicated. The nucleotide positions of the two untranslated regions (UTR) and ORF1ab are shown. The two arrows highlight the recombination hotspots in the S (number 1) and ORF3a (number 2) genes. Please note that the N gene overlaps with ORF9b and ORF9c (not shown). NCBI Reference Sequence: NC_045512.2 The 5′-Cap and 3′-polyA tail are not shown.

3. The Emergence of new variants

3.1. The appearance of dominant variants is preceded by minor lineages

As shown in Figure 1, novel lineages evolve from the pool of intra-host variations (iSNVs) at different times in different geographical regions. Minor mutations become SNPs when they help to escape the immune system, widen host tropism or enhance receptor binding. This concept is illustrated by a study conducted in Norfolk (UK) during the first wave of the pandemic between March and August 2020. The analysis of 1035 viral genomes revealed 26 distinct global lineages that were introduced into the region by

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travel as well as 100 minor lineages specific to the UK. Interestingly, the geographical distribution of the minor lineages was in some cases very restricted. For example, 35 infections in a food processing plant were caused by the minor UK lineage UK1361 that could not be detected in the surrounding communities [13].

Similar observations were made during the first 15 months (January 2020 till March 2021) of the pandemic in North America [37]. The disappearance of the Wuhan lineage between March and May 2020 was followed by a mutational burst in the summer of 2020 from which 14 predominating lineages emerged. Towards the end of 2020, a number of low frequency mutations emerged within these 14 lineages amongst them were two N-terminal deletions (ΔH69, ΔV70) and two missense mutations (N501Y, D614G) in the Spike receptor that became later the defining signature of the Alpha variant (B.1.1.7) which eventually dominated the patient samples in 2021 [37]. Importantly, the mutational bloom in the summer of 2020 was preceded by the appearance of the D614G mutation in Spike and the P323L substitution in the RNA-dependent RNA Polymerase (RdRP, NSP12). The combination of both mutations probably ensured the extraordinary epidemiological success of the D614G mutation that is now present in all known variants after the demise of the Wuhan strain [38]. While the D614G change enables the virus to conquer new cells more effectively as the Spike receptor adopts its open conformation more frequently [7], the P323L replacement in RTC may have allowed the D614G genomes to move more effectively through bottlenecks given the role of RdRP in the amplification of the viral RNA.

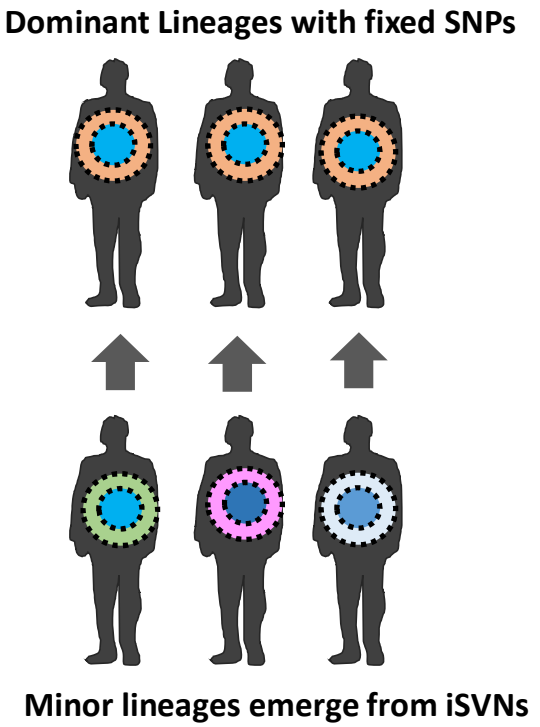

Figure 3: The emergence of dominant variants is preceded by minor lineages. Minor lineages evolve in geographically restricted areas from intra-host variants (iSNVs) with enhanced fitness. Once a combination of minor mutations gains dominance in a population and is repeatedly sampled, it is recognized as a novel lineages or variant. The initially rare iSNVs became fixed and are now population-wide single nucleotide polymorphisms (SNPs).

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Lineage defining mutations that appeared first as minor mutations in local communities were also observed in India between November 2020 and May 2021 prior to the emergence of the Kappa (B.1.617.1) and Delta (B1.617.2) variants [39] [40]. A possible reason why the Indian subcontinent was so dramatically affected may have been the high prevalence of the A97V (SNP: C13730T) and P323L (SNP: C14408T) mutations in the RNA-dependent RNA polymerase (NSP12) on the subcontinent [39]. Both substitutions correlate with an approximately 100-fold higher evolution rate in India (6,73 x 10^{-2} substitutions/site/year) compared to the global average (6,50 x 10^{-4} substitutions/site/year) [41].

These observations inform a model such that minor lineages with novel fitness enhancing mutations circulate first in small clusters or communities within restricted geographical locations before they further evolve to become the dominant lineage that is then spread by travel to other regions of the world (Figure 3).

3.2. The origin of Intra-host variants (iSNVs)

An analysis of 1181 samples collected in East England in 2020 revealed 11,216 within-host variants (on average 9,5 iSNVs per sample) containing 7102 single-nucleotide substitutions, 3223 small deletions, 542 small insertions and 349 multi-nucleotide variants [9]. The most intriguing observation, also made my others, was the dominance of cytidine to uridine transitions (C→U; 47,9%) in the 5′-3′sense strand while the opposite change from guanosine to adenosine (G→A) in the 3′-5′anti-sense strand accounted for only 5,3% [9] [37] [42] [43]. Since this strand asymmetry is more pronounced in SARS-CoV-2 compared to SARS-CoV-1 and MERS, and absent in bat coronaviruses [42], it may arise from the replication of the virus in human cells. Given the strand asymmetry, it is unlikely that the mutations occur during RNA replication since the error rate of RdRP/NSP12 is the same for both strands. For this conclusion speaks also the completely different mutation profile of an RdRP mutant lacking its proofreading function (NSP14 deletion) [44].

The likeliest source of the C→U substitutions are enzymes of the human APOBEC family which deaminate cytidines to uridine (C→U) in single-stranded RNA at cytoplasmatic RNA processing centers, stress granules and p-bodies [45] (Figure 4). APOBEC deamination is part of the anti-viral defense as the additional C→U mutations can push RNA viruses over the error threshold. Unlike SARS-CoV2, most RNA viruses lack a proofreading function and replicate therefore with a high mutation rate close to a level that would compromise their survival. This mutation level is the error threshold [46]. The ability of SARS-CoV-2 to highjacked this anti-viral defense for its evolution lies in the proofreading function of its 3′-5′exoribonuclease NSP14. This reduces its replicative mutation rate to 1-3x10^{-6} mutations/nucleotide/replication cycle, which is at the lower end for ssRNA viruses (10^{-4} to 10^{-6} mutations/nucleotide/replication cycle) [12] [46].

Intriguingly, the high C→U deamination rate correlates with an elevated ratio of nonsynonymous (amino acid changing) to synonymous substitutions (dN/dS). This ratio is unusually high for SARS-CoV-2 (0,65 – 0,728) when compared to SARS-CoV-1 (0,428), MERS (0,228) and bat corona viruses (0,096 – 0,155) [42]. Hence, every second codon change may therefore result in an amino acid substitution. This is reflected at the level of iSNVs where on average 75% are nonsynonymous and 25% are synonymous [37] [42].

The second most frequently observed base substitution (14,3%) are guanosine to uridine (G→U) transversions in the 5′-3′sense strand [9] (Figure 4). As in the case of the APOBEC enzymes, the increase in G→U substitutions is not found in bat coronaviruses [47]. The likeliest cause of the G→U mutations are reactive oxygen species (ROS). RNA is more prone to oxidation than DNA, guanine is the most oxidation-sensitive base and, interestingly, oxidation is suppressed in bat cells [48]. The mechanism may involve the oxidation of guanine to 8-oxo-guanidine which pairs with A in the antisense RNA thereby replacing guanine with an uracil in the subsequent replication round. Approxi-

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mately 75% of the G→U changes cause amino acid changes [47]. Intriguingly, elevated ROS levels are found in cells with high APOBEC activity [49].

The third group of substitutions are A→G transitions at a rate of about 9% [50]. A→G changes are attributed to the family of human ADAR enzymes that act on the double-stranded RNA intermediate during viral replication where they deaminate adenine to inosine. Since inosine pairs like guanine, the uracil in the 3′-5′antisense strand is replaced by a cytosine. [51] (Figure 4).

Taken together, SARS-CoV-2 appears to joyride on the anti-viral defense mechanisms to drive its evolution in a way that is not found in bat coronaviruses and to a much lesser extend in SARS-CoV-1 and MERS.

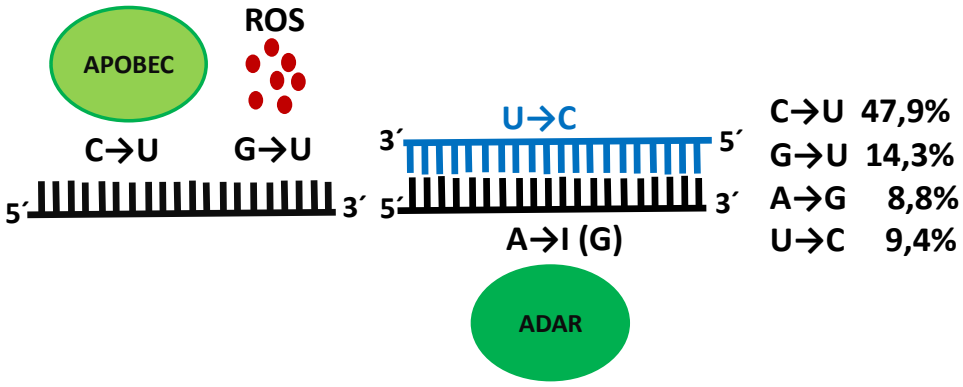

Figure 4: Mutation mechanisms affecting the viral RNA. Proteins of the APOBEC family deaminate cytidine to uridine (C→U) in single-stranded RNA. This process affects mainly the 5′-3′ sense RNA while similar changes in the 3′-5′antisense RNA are infrequently found. Reactive Oxygen Species (ROS) modify guanosine to 8-oxoguanosin that pairs with adenosine causing a G→U change in the subsequent replication cycle. ROS levels may rise in the response to APOBEC induced RNA damage. ADAR enzymes deaminate adenosines (A) to inosine (I) that pairs like guanosine (G). Since ADAR enzymes act on the double-stranded RNA replication intermediate, A→G changes in the sense and U→C changes in the antisense strand are balanced. The corresponding frequencies of these changes are shown [9] [50].

A study of 402 clinical samples from 170 patients in China in April 2020 revealed some interesting observations about iSNVs [52]. The average density of iSNVs across the SARS-CoV-2 genome was found to be 0,53 iSNVs per kilobase or 15 iSNV per genome with a two-fold increase in both UTR regions, in ORF8 and in the N gene. No mutation bias was detected between the three codon positions. When samples were collected from the same person for up to 5 weeks, the average iSNV density rose from 0,53/kb to 0,85/kb. Unexpectedly, this increase was higher in patients with mild symptoms, in younger patients and in females. While 81% of these iSNVs were unique to the patient, only 16 out of 7037 iSNV (0,2%) were identified in at least 15 individuals. This may explain why only ~20% of the nonsynonymous iSNVs were later (May-December 2020) found in the 2019nCoVR database meaning that they traversed successfully through bottlenecks before they were fixed as SNPs.

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3.3. The high mutation rate in the Digestive System

The mutational burst observed in the USA during the summer of 2020 [37] is intriguing as it happened while the Covid19 cases were rather low during a time when people were outdoors. Where the virus hibernates during such low transmission periods is as yet unknown. One possible evolutionary refuge is the digestive system (Figure 5). Infectious viral RNA can be detected in the feces of approximately 40% - 50% of mild and server Covid-19 patients within two weeks of diagnosis and up to 4-5 weeks after the virus has cleared from the respiratory system [53] [54] [55]. The viral load can reach 10^7 copies/g feces exceeding the respiratory load (i.e. 10^5 copies/g) [56] [57]. Importantly, the iSNV diversity is more than 10-fold higher in fecal samples compared to the respiratory tract. It also worth noting that the iSNV profiles do not overlap between organ systems suggesting independent modes of evolution [8]. Viral replication in enterocytes was confirmed with human small intestinal organoids (hSIOs) and is in line with the high Ace2 expression level in the digestive system (small intestine, colon, duodenum) [58] [59]. A longitudinal wastewater study analyzing viral RNA from 14 water treatment plants in New York between January and June 2021 detected cryptic lineages with a high geographical restriction that were distinct from the largely respiratory samples in the GISAID database. Importantly, between Mai and June 2021 some of these cryptic lineages dominated the sequence samples reminiscent of the mutational burst in the USA one year earlier in summer 2020. The high geographical restriction agrees with the notion that these lineages evolved in the digestive system of people living in these areas. An animal reservoir like cats, rodents or dogs can however not be ruled out [60].

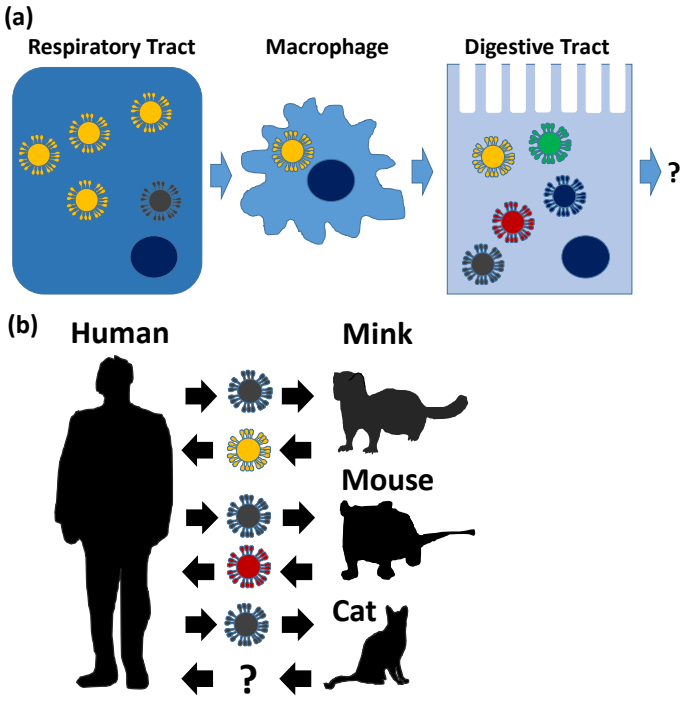

Figure 5: Evolutional reservoirs. (a) Hiding inside macrophages or leukocytes, the virus may travel from the respiratory tract to the enterocytes in the digestive system. The diversity of intra-host single nucleotide variants is approximately 10-fold higher in the digestive system where the virus actively replicates and impacts on the gut ecosystem [61]. Whether active virus in stool can return to humans remains to be confirmed. (b)

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Reciprocal infections between humans and mink has been reported. The Spike mutation Y453F evolved in mink before its transfer to humans [62]. While domestic cats can be infected by their owners [63], it is still unclear whether cats can pass the virus back into the human population. Recent evidence suggests a role of mice in the evolution of the Omicron variant.

While the digestive system may provide an evolutionary refuge for the virus, it is not yet clear whether and how the virus can infect people after its excretion. Despite some case reports [64], direct evidence of fecal–oral transmission is still missing. It remains also to be explored how the virus travels from the cells in the respiratory tract to the enterocytes in the digestive system. One possible explanation offers the detection of viral RNA in migrating lymphocytes and macrophages [65].

3.4. Mutation in an animal reservior

Reciprocal transmission between human and animal hosts was first observed in June 2020 in Denmark where the new variant B.1.1.298 emerged in the vicinity of mink farms with the unique Y453F mutation in the Ace2 binding domain of Spike [62] (Figure 5). Although this is so far the best case study of human-animal-human transmission, the Spike receptor has a broad tropism for different mammalian species in vitro. Using Spike from the alpha (B.1.1.7) variant, the authors reported the highest affinity for Ace2 from domestic cats, dogs and cattle while Ace2 from bats, birds and rats displayed the lowest binding strength [66]. Intriguingly, the tropism spectrum of Spike significantly widened during the successive waves of the pandemic due to the appearance of the mutations K417T, E484K and/or N501T in its Ace2 binding domain. While the Wuhan strain fails to bind to Ace2 from mice, all subsequent VOCs efficiently infect murine cells in vitro [67]. Given the high similarity between feline and human Ace2, domestic cats are a possible animal reservoir where the virus might hibernate and mutate. For example, feces samples of an infected cat living with an infected owner taken on two dates 7 days apart contained viral RNA that differed at 4 and 14 positions from the original delta variant (B.1.617.2 AY.3) [68]. Whether such mutated strains can however re-infect humans has not yet been reported although viral shedding and transmission between cats was observed [63] [69].

Interestingly, recent evidence suggests a central role of mice in the evolution of the omicron variant (B.1.1.529). Its long evolutionary distance from all other variants and the unusually high number of mutations (average 53,3) led to speculations about an as yet unknown animal host in which the virus may have evolved. Intriguingly, early omicron genomes lack the high C→U deamination rate which is the hallmark of human APOBEC RNA editing. Also the mutation rate in the spike genes was with ~1,5 mutations per month three times higher when compared to the S gene from the other variants [70]. Since two of the mutations in the hAce2 binding domain of Spike (Q493K, Q498H) significantly enhance the affinity towards mouse Ace2 [14] and were also found in experiments were the human SARS-CoV-2 virus was repeatedly propagated in mice [70], it might be possible that the progenitor genome of omicron transferred to mice were it rapidly evolved before returning to humans (Figure 5).

3.5. Evolution of the virus in immune-compromised patients

The increase in iSNVs over time in infected patients [52] implies an important role of co-morbidities in the evolution of novel variants. A case study with three severely immunosuppressed patients suffering from a follicular lymphoma revealed however a very diverse picture. While the 12 iSNVs in patient A became quickly fixed within 13 to 15 days after sampling started, this was not the case for the remaining two patients. Pa-

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tient B developed with 28 more iSNVs but the duration to fixation was between 1 and 2 months. Finally, patient C evolved only 4 iSNVS which became fixed again after 1-2 months [71].

The longitudinal analysis of samples over 140 days obtained from an immunosuppressed patient with a history of autosomal dominant polycystic kidney disease (ADPKD) found an interesting delay in the appearance of iSNVS. While no iSNVs were detected in the first 14 days, minor mutations started to appear from day 42 onwards [72]. While some genetic alterations were only present at a singular time point, others became fixed revealing the existence of two novel lineages in this patient after 105 days. In one lineage, the deletion of amino acids 141-144 in the N-terminus of Spike was linked with the mutation F490L in its hAce2 binding site. In the second lineage, the N-terminal deletion aa 244-247 was linked with the E484G mutation in the hAce2 binding site. The emergence of these linages support the argument that immune compromised hosts play an important role in the evolution of SARS-CoV2. This particular case is especially fascinating since nonsynonymous replacements of glutamate 484 are present in most important variants including Gamma (P.1; E484K), Beta (B.1.351; E484K), Delta (B.1.617; E484Q) and Omicron (B.1.529; E484A). Moreover, the two N-terminal deletions affect the loops N3 (aa 140-158) and N5 (aa 245-264) in Spike to which many neutralizing antibodies bind [73]. A nonsynonymous replacement of E484 was independely detected 75 days and 128 days post-diagnosis in a patient suffering from the immune disorder antiphospholipid syndrome [74].

3.6. The role of recombination

The recent discovery of the "deltacron" variant in February 2022 in France swung the spotlight on the ability of single-stranded RNA viruses to exchange genomic information through recombination [75].

Recombination is an important tool for RNA viruses to remove fataly mutated genomes and to generate sub-genomic templates for the translation of the structural genes. Since most RNA viruses replicate close to the error threshold, the recombination between two inactive templates can generate a functional genome. Two distinct mechanisms allow to combine genetic information from different RNA molecules.

A replication-dependent template switch (also known as copy-choice) occurs when the viral RNA-dependent RNA polymerase NSP12 stalls at a secondary structure or defined sequence motive only to restart RNA synthesis after having switched to a new template (Figure 6) [76]. When the restart happens at the homologe position on the new template no sequence information is lost (precise recombination). In the case of a restart downstream of the switch point, genetic information is deleted (imprecise recombination). Imprecise recombination can also happen within the same RNA template (Figure 6). In the case of SARS-CoV-2, this produces subgenomic RNAs where the long ORF1ab region is deleted to favour translation of the structural genes. The RdRP (NPS12) polymerase is necessary for the template switch, and the swiching rate negatively correlates with its replicative fidelity [77]. In the case of SARS and MERS viruses, the proofreading exoribonuclease (Nsp14) within the replication-transcription compex plays an active role in this process as its inactivation lowers the recombination rate [78]. The SARS-CoV-2 genome harbours several known recombination hotspots in the spike gene and in the genes Nsp2, M, N and ORF8 which may contain sequence motives that favour NPS12 stalling and template switching [79]. Replication-dependent template switching is the main recombination mechanism.

A minor pathway is the replication-independent mechanism that re-joins overlapping fragments of the viral RNA [76]. Since translation of the viral genes is no pre-requisite for this process [80], it may entirely depend on host factors. How important this mechanism really is for viral evolution is still under discussion [81].

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In the case of the deltacron genome (NCBI sequence: OM991095), template switching occurred between the delta (B.1.617.2 21J/AY.4) and omicron variant (B.1.1.529 21K/BA.1) in a cell that was infected by both lineages (Figure 7). While the delta genome forms the backbone, a large section of the spike protein and the beginning of ORF3a comes from the omicron strain [82]. This section contains 27 mutations spanning from Asparagine-211 in the N-terminal domain of Spike to Leucine-981 in its S2 C-terminal section with 14 amino acid substitutions affecting its Ace2 binding region [83]. A second "deltacron" variant was identified in Januray 2022 in the USA [84]. The interesting observation is here that almost the same recombination hotspot at the beginning of the Spike gene was utilised in both independenty evolved lineages (USA isolate: 22,036-22193nt; French isolate: 22,034-22194nt). However in contrast to the french variant, the american strain has only one cross-over point so that the entire 3′-section comes from the Omicron strain (Figure 5).

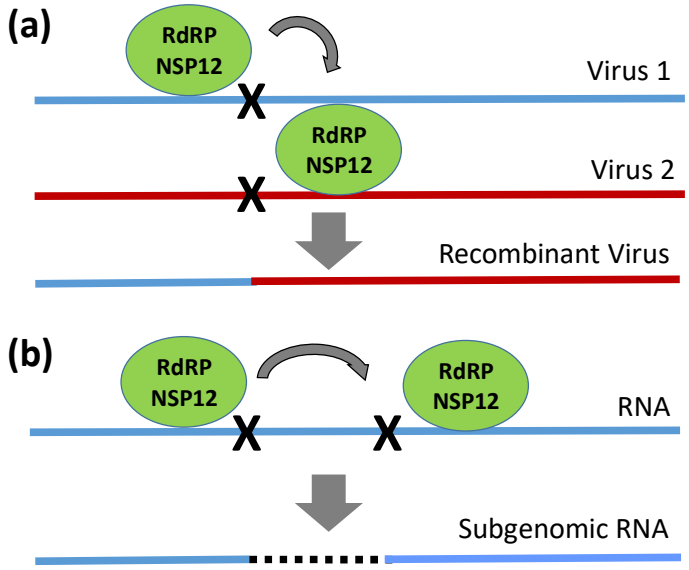

Figure 6: Replicative Recombination and synthesis of sub-genomic RNAs. (a) At secondary structures or specific sequence motives (indicated by the letter X), the RNA-dependent RNA Polymerase (RdRP) switches from one RNA template to a second RNA template. If this second RNA template stems from a co-infecting SARS-CoV-2 virus, a hybrid virus is generated. This copy-choice mechanism is also used to purche cells from terminally mutated RNA genomes by generating a functional RNA from two non-functional templates. If the RdRP re-enters the second template at the same position, no sequence information is lost (precise recombination). (b) During viral replication, RdRP switches position on the same RNA template. This results in a sub-genomic RNA where sequence information has been deleted (indicated by the dotted line) (imprecise recombination) *[76]*

The first report of the local transmission of a hybrid virus came however from the the UK where 16 recombinant lineages were found in 279,000 sequences up to March 2021 [85]. Twelve of these lineages clustered into 4 groups which originated in distinct geographic localities. While most of these sequences obtainend the spike information from the B.1.1.7 alpha variant, the remaining genomes came from different non-B.1.1.7

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lineages. Intriguingly, these lineages circulated in the same geographic area as the recombinant strains immediately prior to their detection.

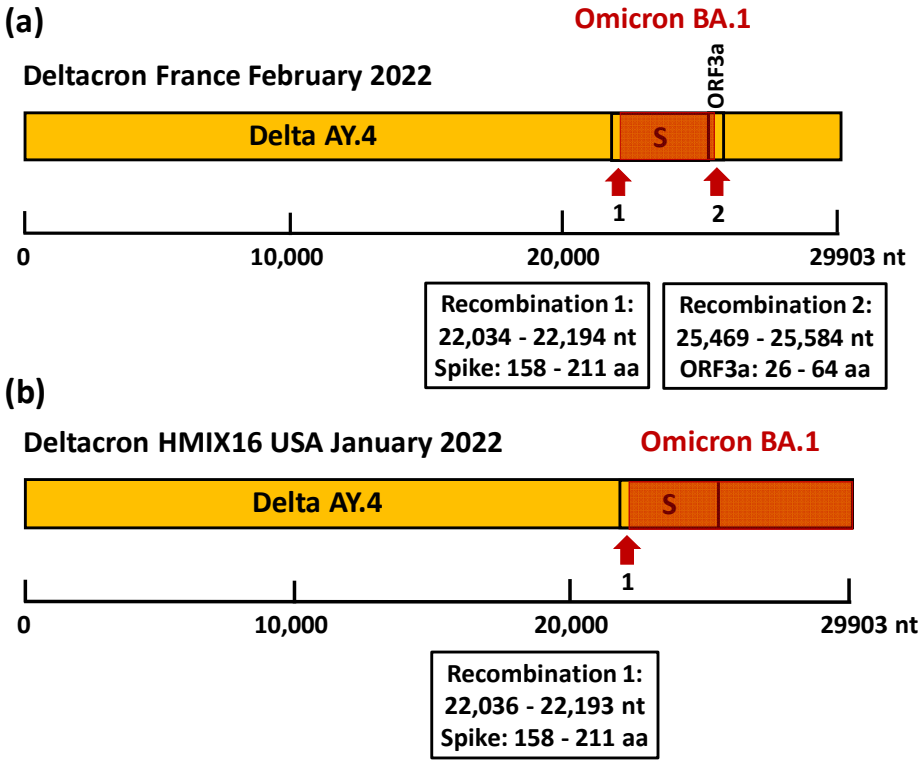

Figure 7: The Deltacron lineages. (a) The Deltacron lineage discovered in France in February 2022 caries most of the Spike gene and the beginning of ORF3a from the Omicron 21K/BA.1 lineage while the rest of the genome corresponds to the Delta 21J/AY.4 strain [82]. The arrows highlight the recombination cross-over regions. (b) The Deltacron lineage isolated in the USA in January 2022 possesses only one cross-over point between Omicron 21K/BA.1 and Delta 21J/AY.4. This results in more Omicron sequence till the 3′-end of the genome. Please note that the recombination region 1 of both independently isolated lineages almost match.

4. Conclusions

Novel variants do not pop up one-day infecting patient zero, they rather evolve gradually from cryptic lineages initially circulating in small clusters or local communities where they acquire fitness-promoting mutations from the pool of intra-host variations. Migration between organ systems while hidden in leucocytes or macrophages may help to take advantage of the elevated mutation rate in the digestive tract. Anti-viral defense mechanisms in form of reactive oxygen species, APOBEC and ADAR enzymes are harnessed to generate intra-host mutations. Their frequency and selection is promoted by certain comorbidities like immune-suppression. Reciprocal human-animal transmissions enable the virus to take advantage of higher selection pressures in the new host thereby accelerating its evolution rate. While recombination occurs frequently amongst RNA templates in the same cell, hybrid lineages like deltacron may be seen more often as the rate of coinfection increases.

Funding: This research received no external funding.

Conflicts of Interest: The author declares no conflict of interest

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