

1 **Preliminary rapport on SARS-CoV-2 spike mutations arising in Danish mink,**
2 **their spread to humans and neutralization data.**

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4 **SARS-CoV-2 spike mutations arising in Danish mink and their spread to**
5 **humans**

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8 **Background**

9 Despite control measures, SARS-CoV-2 continued to spread among mink farms across northern
10 Denmark, with more than 200 farms infected by November 2020. SARS-CoV-2 genome sequences
11 obtained from infected mink and humans living on the farms provided evidence of SARS-CoV-2 spread
12 between mink and human in zoonotic events. This study investigates the amino acid changes in the
13 spike surface glycoprotein that appeared during this outbreak and their effect on the antigenicity of
14 the SARS-CoV-2 virus.

15 **Spike mutations**

16 Within the infected mink, the SARS-CoV-2 virus mutated, giving rise to several amino acid changes in
17 the spike protein. The first was a tyrosine to phenylalanine at amino acid 453 (Y453F), a mutation that
18 also appeared during the Dutch mink farm outbreaks. It is a conservative amino acid substitution in
19 the receptor binding domain that directly contacts the host ACE2 receptor at amino acid 34 (Wang et
20 al). This ACE2 contact position differs between human and mink (histidine [34H] in humans and
21 tyrosine [34Y] in mink and other mustelids (Damas et al)), which suggests that Y453F is an adaptation
22 mutation to mink ACE2. Importantly, 453F increases affinity for human ACE2, which may explain its
23 successful introduction and establishment in humans.

24 Following the appearance of 453F, additional spike mutations were observed in minks and the humans
25 epidemiologically linked to the infected mink farms (Fig. 1). These include: i) 69-70deltaHV - a deletion
26 of a histidine and valine at amino acid positions 69 and 70 in the N-terminal domain of the S1 subunit;
27 ii) I692V – a conservative substitution at position 692 that is located seven amino acids downstream
28 of the furin cleavage site; iii) S1147L – a non-conservative substitution at position 1147 in the S2
29 subunit; and iv) M1229I – a conservative substitution located within the transmembrane domain.

30 **Clinical isolates**

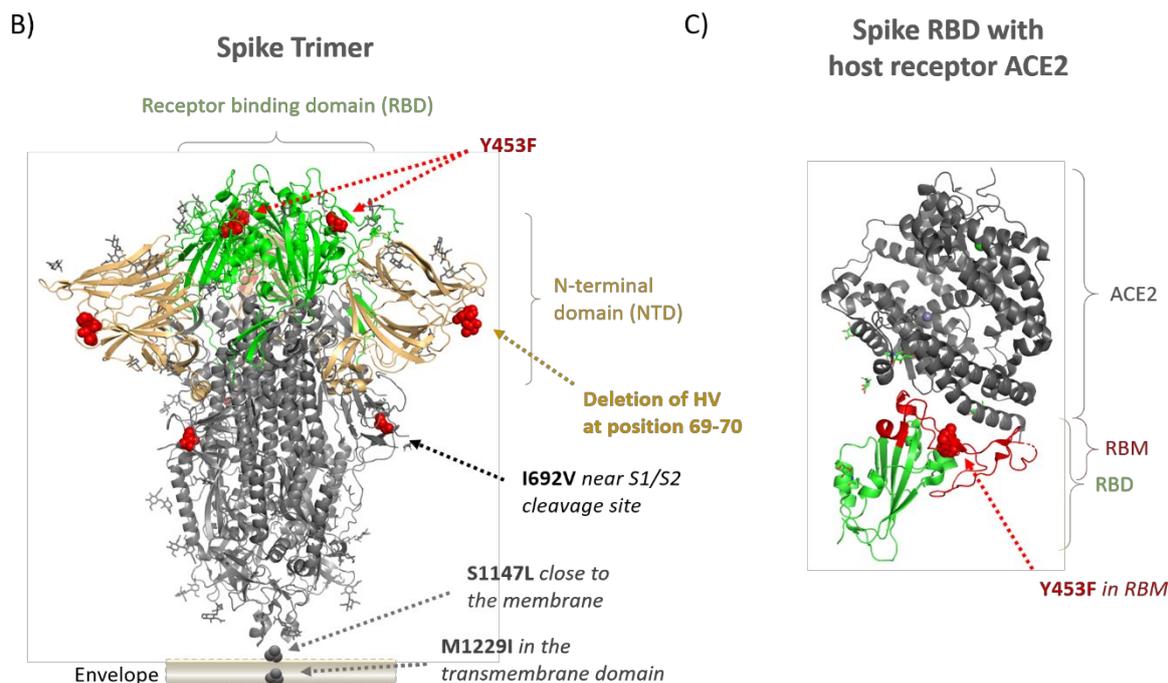
31 Efforts are underway to isolate each mink-associated SARS-CoV-2 spike mutant strain that occurs in
32 people residing in Denmark. To date, Statens Serum Institut in Denmark has isolated two strains of
33 mink-associated SARS-CoV-2 viruses. These include an isolate with the 453F spike mutation (F-spike)
34 from cluster 1 and an isolate with a 69-70deltaHV, 453F, 692V, and 1229I mutation combination from
35 Cluster 5 (hereafter referred to as Δ FVI-spike). To ensure that subculturing of SARS-CoV-2 clinical
36 isolates on VeroE6 cells did not induce additional spike mutations, each isolate was sequenced. The
37 spike protein of the cultured virus was identical to that of the SARS-CoV-2 virus in the original clinical
38 sample.

A)

Spike mutation combinations*	Abbreviation	Number of positive clinical samples**
453F	F	N = 142
69-70delHV, 453F	ΔF	N = 162
69-70delHV, 453F, 1147L	ΔFL	N = 18
69-70delHV, 453F, 692V, 1229I	ΔFVI	N = 12

* All SARS-CoV-2 mink-associated sequences also contained the D614G

** For sequenced samples up until 31 October 2020. May include duplicate samples taken from the same person and is therefore not necessarily representative of the number of infected persons.



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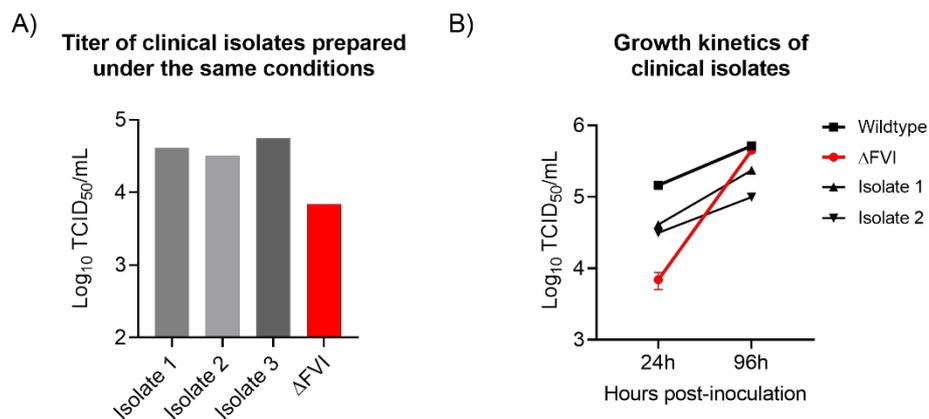
40 **Figure 1. The mink-associated mutations in the SARS-CoV-2 spike protein.** A) The combination and frequency
 41 of mink-associated spike mutations detected in SARS-CoV-2 infected humans B) The crystal structure of a closed
 42 prefusion spike trimer [PDB: 6ZGE] with the position of the Y453F variant in the receptor binding motif, the
 43 position of two amino acids deleted in the N-terminal domain, and the position of the I692V variant. The regions
 44 encompassing the S1147L and M1229I mutations are not within the crystal structure; however, their relative
 45 positions are indicated. C) The position the Y453F variant in a receptor binding domain complexed with a host
 46 ACE2 receptor [PBD: 6LZG].

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48 The clinical isolates bearing the Y453F spike mutation replicated as efficiently as the
 49 unmutated/wildtype SARS-CoV-2 virus that predominates in Denmark (data not shown). Conversely,
 50 the SARS-CoV-2 virus with four mutations grew slower than both the wildtype virus and other SARS-
 51 CoV-2 virus isolates (Fig. 2). The cytopathic effect (CPE) induced by the ΔFVI-spike mutant virus
 52 appeared later and was less pronounced and had an approximate 10-fold lower titer 24 hours post-
 53 inoculation compared to human SARS-CoV-2 isolates prepared under the same conditions (Fig. 2A). At
 54 96 hours post-inoculation the ΔFVI-spike mutant virus titer was comparable to that of the wildtype

55 virus and exceeded other SARS-CoV-2 viruses isolated and subcultured under the same conditions (Fig.
56 2B). The Δ FVI-spike mutant virus titer increased 54.7-fold from 24 to 96 hours post-inoculation,
57 compared to an average of 4-fold (range: 2.6 to 5.7-fold) over the same time for other SARS-CoV-2
58 isolates. The ability to replicate to high viral titers is consistent with high levels of the Δ FVI-spike
59 mutant virus detected in throat swab samples of infected persons, as indicated by an average qPCR
60 assay (E-Sarbeco) cycle threshold of 24.7 (range: 20-35). Further evaluation of the SARS-CoV-2 Δ FVI-
61 spike strain growth kinetics in other cells systems are warranted.

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64 **Figure 2. Growth kinetics of the SARS-CoV-2 Δ FVI-spike mutant virus.** A) Virus titers 24h post-inoculation for
65 SARS-CoV-2 viruses isolated from clinical samples under the exact same conditions. Isolate 1-3 each have
66 different spike mutations unrelated to mink outbreaks, these include N439K (isolate 1), N439K+69-70delHV
67 (isolate 2), and S477N (isolate 3). B) The growth kinetics of the Δ FVI-spike mutant virus relative to other clinical
68 isolates, including the nonmutated virus (wildtype) that predominates in Denmark and spike mutant viruses
69 (isolate 1 and 2 as for [A]).

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71 Virus neutralization

72 The introduction of SARS-CoV-2 spike mutant viruses raises concerns about a potential reduced
73 recognition of the protein by antibodies induced after SARS-CoV-2 infection or vaccination that may
74 have implications for re-infections and vaccine efficacy, respectively. To evaluate the effect of the
75 mink-associated SARS-CoV-2 spike mutant viruses on antigenicity, neutralizing activity of convalescent
76 plasma from persons who recovered from a SARS-CoV-2 infection and sera from immunized rabbits
77 were compared between the Δ FVI-spike mutant virus and an unmutated wildtype virus.

78 The neutralization activity was tested using a micro-neutralization assay that was adapted from the
79 World Health Organization protocol for influenza virus neutralization. The assay was developed at
80 Statens Serum Institut and validated on >300 convalescent plasma/serum samples as well as sera from
81 vaccinated mice and rabbits. In brief, 2-fold serial dilutions of plasma/sera were pre-incubated with

82 SARS-CoV-2 virus for 1 hour before addition to a monolayer of VeroE6 cells prepared in 96-well plates.
83 After a 24 hour incubation, the cells were fixed to the plates and the level of virus determined using a
84 standard ELISA targeting the SARS-CoV-2 nucleocapsid protein. To determine the amount of virus to
85 add to the assay, clinical isolates are usually titrated at 24 hours and from these titers $100\times$ TCID₅₀
86 virus used in the neutralization assay. This equates to approximately $300\times$ TCID₅₀ from titers calculated
87 96 hours post-inoculation. Due to the difference in growth kinetics of the Δ FVI-spike mutant virus, the
88 TCID₅₀ titer calculated at 96 hours was deemed to reflect the amount of infectious particles in the virus
89 stock more accurately than that measured at 24 hours post-inoculation. Thus, each serum samples
90 were tested in duplicated with $300\times$ TCID₅₀ as calculated from 96 hours post-inoculation titers.

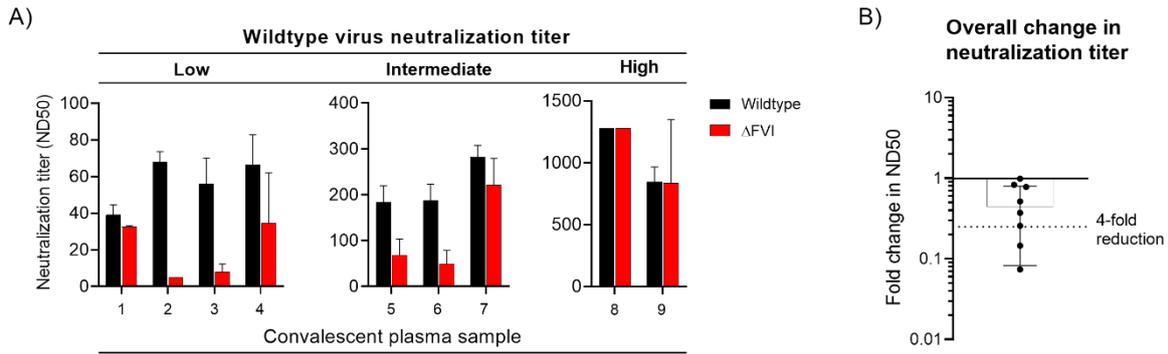
91 The convalescent plasma was selected from persons living in the South of Denmark, geographically
92 separated from the mink outbreaks in the North of Denmark, and had a documented SARS-CoV-2
93 infection at the beginning of the Danish epidemic before the mink outbreaks occurred. Since the effect
94 of the spike mutations on different levels of neutralizing antibodies is unknown, sera with known low
95 (N=4), intermediate (N=3) and high (N=2) neutralization titers were tested. Each plasma sample
96 represents a different donor and was tested in duplicate.

97 The different convalescent plasma were not equally affected by the Δ FVI-spike mutant virus. The two
98 plasma samples with high neutralization titers were largely unaffected, while plasma with low and
99 intermediate titers were more likely to experience a loss in neutralization activity (Fig. 3a). In these
100 preliminary data from 9 convalescent plasma, an average 3.58-fold (range: 0 to 13.5) reduction was
101 observed. Only two plasma samples had a greater than 4-fold reduction, a threshold set for
102 neutralization resistance by Li et al. who evaluated other spike mutants presented on pseudovirus
103 particles. It is important to note that the findings are preliminary and warrant further investigation in
104 other SARS-CoV-2 neutralization assays.

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109 **Figure 3. Neutralization of the SARS-CoV-2 Δ FVI-spike mutant virus relative to an unmutated SARS-CoV-2**
110 **virus.** A) Convalescent plasma from nine individuals with known low, intermediate, or high neutralizing titers
111 were used to assess the effect of the spike mutations on neutralization activity of antibodies induced following
112 infection with an unmutated SARS-CoV-2 virus. The neutralization titer was determined as follows: a 50% cut-
113 off value was calculated using quadruplicate virus controls (prepared for each virus) and cell controls included
114 on each plate. The titer was calculated as the interpolation of a 5-parameter titration curve with the 50% cut-
115 off value. The reciprocal serum dilution is reported as the 50% neutralization antibody titre. B) The fold-change
116 in neutralization titer for the SARS-CoV-2 Δ FVI-spike mutant virus relative to an unmutated SARS-CoV-2 virus.
117 The horizontal dotted line indicates a 4-fold reduction. The bars represent the mean of duplicate measurements
118 with the standard deviation.

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122 **PRELIMINARY References**

123 Wang et al (2020) Structural and Functional Basis of SARS-CoV-2 Entry by Using Human ACE2

124 Damas et al (2020) Broad host range of SARS-CoV-2 predicted by comparative and structural analysis
125 of ACE2 in vertebrates

126 Li et al (2020) The impact of mutations in SARS-CoV-2 spike on Viral Infectivity and Antigenicity. Cell
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